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# HANDBOOK OF BACTERIOLOGY

For Students and Practitioners of Medicine

BY

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DEDICATED

TO THE MEMORY OF

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TEACHER, COLLEAGUE, FRIEND



## PREFACE TO THE FIFTH EDITION

My object, in writing this Handbook, was to supply accurate information on bacteriology in a form suitable for students of medicine. The use of this book in schools of medicine in various parts of the world and the fact that it has now reached its fifth edition appear to prove that I have succeeded in being of assistance to the student, and such success cannot fail to be gratifying to even the most modest author.

Three and a half years have elapsed since the publication of the fourth edition of this Handbook and, during this time, definite advances in bacteriology have been made in a variety of directions. I have endeavoured to keep the Handbook up to date by incorporating in it all new work which appeared to me to be sound and of sufficient importance to the student of medicine. Such additions would have increased the size of the book considerably but for rigorous excision and condensation of less important matter, as a result of which the text of the new edition occupies only twelve more pages than did that of its predecessor.

The greatest changes have been made in the chapters on Antiseptics, Immunity, Streptococci, Food-poisoning and Dysentery Bacilli, Undulant Fever and Tubercle Bacilli, but actually only one-quarter of the pages remain without some alteration.

In this edition I employ, for the first time, some of the newer names now commonly applied to bacteria. The majority of these are derived from Bergey's "Manual of Determinative Bacteriology," but I have not invariably followed Bergey's lead, sometimes preferring the more conservative nomencla-

ture of Topley and Wilson. Many bacilli are temporarily grouped together in the provisional genus *Bacterium* until such time as more general agreement is reached as to how this large genus should be subdivided.

The number of illustrations in the text has been increased by seven and many of the older illustrations have been replaced by new drawings and photographs prepared by Mr. W. Kampff. I am greatly indebted to Dr. C. E. van Rooyen for the photographs used for Figs. 82 and 83, and to Dr. E. C. Smith for the drawing used for Fig. 36.

It is with great pleasure that I acknowledge my gratitude, for helpful criticisms and suggestions and for much assistance during the task of revision and proof reading, to my colleagues, Dr. R. A. Q. O'Meara, Dr. W. Gillespie, Dr. E. Marjorie Booth, Dr. W. Hayes and Dr. C. Mushatt.

I trust that this, the fifth edition, will be appreciated not only by teachers of Bacteriology but also by students of the subject, for whom it has been written.

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## PREFACE TO THE FIRST EDITION

THE publication of a new Handbook of Bacteriology may seem to demand, if not an apology, at least an explanation, in view of the large number of such works available.

This volume is the outcome of the dissatisfaction with the existing text-books, frequently expressed by students to the author. The complaint is that the text-books contain seven to eight hundred pages, and the student has not sufficient time to master their contents in the few months devoted to the study of Bacteriology in the medical curriculum, and further that he is unable to distinguish the more important matter from the less. The result often is that the student relies, for examination purposes, on grinders' notes or "cram" books. I feel that this criticism must be taken seriously, since the majority of text-books attempt both to supply the necessary information to the student, and also to act as laboratory reference books. This criticism is not made in any spirit of antagonism to those volumes, to which I have been deeply in debt in preparing this book, and which are constantly on the bench in my own laboratory. I have, however, felt for some years the urgent need of a shorter text-book, suitable for the use of students, and, as no such work appeared, I set myself the task of writing one. Whether I have succeeded or not will be for my readers, particularly my student readers, to say, for it is their need that I have ever had in mind.

This work is primarily intended for students of Medicine, and for those practitioners who have not specialized in Bacteriology. When its contents have been assimilated, I would recommend any one wishing to learn more of the subject to consult one of the larger text-books. I believe that here



the student will find all the more important facts relating to bacteria as they affect man. I have endeavoured, as far as possible, to present the practical aspect of the subject, to reduce the theoretical, and to keep prominently before the reader the fact that bacteria are important to the physician, not in themselves, but only as the causes of disease. Some criticism may be directed to a certain dogmatism in the presentation, but I believe that it is better to be dogmatic, putting forward a probable theory as the correct one, rather than to leave the student confused with the claims of half a dozen rival theories, concerning the relative merits of which only the advanced worker is in a position to decide.

Historical notes are, for the most part, avoided in the text, but beneath the name of each of the more important of the pathogenic bacteria the name of the discoverer and the date are given.

A few words must be said concerning terminology. In the systematic portion of the work the name by which each organism is commonly known is given in heavy type. Following this are some of the alternative names, which are printed in italics. The last of these is, in most cases, the name recommended by the Committee on Determinative Bacteriology of the Society of American Bacteriologists. While some of these names appear strange at first, their adoption will probably become general. These names are usually given as in "*Bergey's Manual*," but in a few cases the spelling has been modified where the Latin seemed doubtful. So "*Hemophilus*" is here written "*Hæmophilus*," and "*gonorrhea*," "*gonorrhœa*."

I have great pleasure in expressing my indebtedness, in writing this book, to the authors of many works on Bacteriology. This indebtedness is not otherwise acknowledged, and so here I gladly record my thanks to the following authors, some living, others dead in the flesh, but still living in the pages of their works—Besson, Hetsch, Hewlett, Hiss, Jordan, Kendall, Kolle, Kolmer, Muir, Park, Ritchie, Stitt, Wassermann, Williams, and Zinsser.

The illustrations are original, with the exception of a few

of certain pieces of apparatus, the loan of the blocks for which is acknowledged beneath them. My thanks are most whole-heartedly given to my friend and colleague, Dr. E. C. Smith, who is responsible for the drawings in the text, and for Coloured Plates I, IV, and V. To Mr. A. E. Terzi I am indebted for Plates II and III, and to my laboratory attendant, Mr. W. Kampff, for the photographs which appear in the text.

For much advice and criticism, for the reading of the manuscript, in whole or part, and for the reading of the proofs, I wish to thank many of my friends and colleagues, among whom I must mention specially Professor J. T. Wigham and Dr. R. H. Micks.

I have endeavoured to eliminate, so far as possible, all errors from this work, but it is probable that some still remain. I would therefore be obliged to any reader who would be good enough to inform me of any errors or incorrect statements which may be observed, in order that these may be corrected, should a second edition be called for.

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# HANDBOOK OF BACTERIOLOGY

## CHAPTER I INTRODUCTION

BACTERIA are minute, rigid, unicellular masses of protoplasm of apparently very simple structure. Their position in the scale of living organisms cannot be regarded as exactly determined, since they possess many characteristics similar to those of both the simpler plants and animals. They may be considered as forming a more primitive type than either of these, intermediate between the yeasts and moulds on the one hand, and the protozoa on the other. They differ from the algæ, which in many respects they resemble, in being devoid of chlorophyll and, in the case of many at least, in the absence of cellulose.

The number of types of bacteria is enormous, since bacteria are present everywhere, but we are concerned only with a limited number, which are associated with disease in man and other animals. It may be said, however, that the popular conception of bacteria as harmful and unnecessary parasites is very far from the truth. It is almost certain that human life on the earth would be impossible were it not for the innumerable activities of bacteria. They form the link between the animal and vegetable kingdoms and render the dead and useless material of the former available for the growth of plants, on which the life of all animals depends. Further,

they fix atmospheric nitrogen and render it available for plant life, which explains the fact that the soil of the earth, suitably tended, is always capable of sustaining vegetation. They are the active agents in many phenomena which are taken so much for granted that the work of the bacteria is often scarcely suspected. The souring of milk, the ripening of cheese, the curing of tobacco, the preparation of leather, and a number of other industrial processes, depend on the action of bacteria.

The bacteria which are responsible for disease in animals are of several different types but, before dealing with these, we may briefly consider their common characteristics. Their size is somewhat varied, but one may say that the average is about 1 micron in diameter. A micron ( $\mu$ ) is  $\frac{1}{1000}$  of a millimetre or  $\frac{1}{254000}$  of an inch. It is obvious, therefore, that in order to be able to see them, a microscope affording a considerable degree of magnification is essential.

When examined in the living condition bacteria are seen to be transparent, colourless, and homogeneous or finely granular. Their presence in liquid, when examined microscopically, can only be detected on account of their refractivity. When killed and suitably stained they are much more easily seen, but very little more information as to their structure is to be made out. No nucleus has ever been satisfactorily demonstrated in bacteria, and it is usually believed that there is no complete differentiation of cytoplasm and nucleoplasm. The endoplasm, that is the central part of the cytoplasm, is probably of nuclear nature and contains grains of chromatin scattered throughout it.

Bacteria are fairly definite in shape, owing to the existence of a cell envelope. This envelope is probably only a specialised part of the ectoplasm and is rarely, if ever, composed of cellulose. In some, the envelope is surrounded by a soft jelly-like material, the capsule, which may have a width greater than the diameter of the bacterium which it surrounds. The presence of capsules often causes a mass of bacteria to adhere together and to behave somewhat like mucus when touched with a wire.

All bacteria require for their growth a considerable amount of water, and they usually flourish, most abundantly, in a moist medium. When observed in the living state, in fluid, some are found to move freely, others to remain motionless. The motility of bacteria is due, in some types, to bending of the whole body of the organism, but more generally to the action of very thin, but relatively long, contractile, hair-like processes, the flagella, with which the motile forms are provided. The motile organisms are chiefly bacilli and spirilla: few of the cocci possess this characteristic. Some bacteria are provided with flagella all around their bodies (peritrichous), others with tufts at one or both ends, and yet others with a single flagellum at each end, or at one pole only. A bacterium provided with flagella is almost certainly motile, even if no movement can be observed at a given time, and the absence of flagella is, except in a few species, indicative of the organism being non-motile. In examining for motility it is necessary to distinguish true motility from the vibratory movement shown even by minute inorganic particles, suspended in fluids, which is known as Brownian movement.

Bacteria multiply, very simply, by fission. They are, so far as is known, entirely asexual. When mature, if there is a sufficiency of food material available, and if other conditions are satisfactory, the individual increases somewhat in size and a constriction appears: this becomes more marked, and finally a septum is produced, which divides the original cell into two individuals, each of which rapidly grows to adult size. The new individuals may separate almost immediately or may remain in contact for a longer or shorter time, depending on the character of their cell envelopes. Under favourable conditions, many bacteria are capable of becoming mature and again undergoing division in from twenty minutes to half an hour. Owing to shortage of food and the accumulation of harmful waste products, this rate of reproduction cannot long be maintained. Even if fission occurred only once each hour, the number of bacteria produced from a single individual, in twenty-four hours, would be almost 17 millions.



Certain races of bacteria, chiefly bacilli, are able to protect themselves against adverse circumstances by the formation of spores. This is not a means of reproduction, since an individual produces not more than one spore and, in doing so, dies. The object is the continuance of the race in conditions which would destroy all the vegetative forms of the bacteria. The spore may be situated at, or near, the middle (central), at one end (terminal), or near an end (sub-terminal).

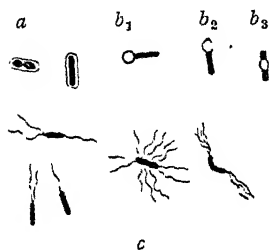


FIG. 1.—CAPSULES, SPORES AND FLAGELLA.

*a*, bacilli with capsules; *b*<sub>1</sub>, terminal spore; *b*<sub>2</sub>, sub-terminal spore; *b*<sub>3</sub>, central spore; *c*, various types of flagella.

Usually a bright, highly refractile spot is first seen in a bacillus; this increases in size until it becomes a round or oval body, shorter than the long axis of the bacillus, but often of greater diameter than the width of the bacillus, in which it produces a bulge. When the spore is fully developed, the body of the bacillus disappears and the spore, which is non-motile, is free. The spore possesses a relatively thick and very resistant membrane, its vital functions are reduced to a minimum, and it is capable of surviving the absence of food and water for long periods, and an exposure to a temperature which would be sufficient to kill the vegetative form of the bacillus in a short time. The great resistance of spores is probably due, in part to their thick cell membrane and to their low water content. The temperature at which solutions of albumin coagulate is greater, the lower the proportion of water in them. When external conditions are again favourable, the spore membrane ruptures and the bacillus itself is reproduced.

The chemical composition of bacteria varies in different species. All, however, consist chiefly of water, which forms from 80 to 85 per cent. of their weight. Of the rest, the greater part is some form of protein material together with carbohydrates, waxes, fats, lipoids, and mineral matter. In addition to carbon, nitrogen, hydrogen, and oxygen, bacteria contain

small amounts of sulphur, phosphorus, calcium, sodium, magnesium, iron, and other elements.

The source of the elements of which bacteria are composed depends very largely on the environment in which they are accustomed to live. We are concerned with those which live in the human or animal body. These types, in common with others, require a source of energy for their growth to enable them to build up the constituents of their protoplasm. For the pathogens, the commonest and also the most readily available source of energy is carbohydrate which, in the form of glucose, is always present in low concentration in the body fluids and the laboratory media made to imitate these fluids. Utilizing a series of reactions very similar to that by which lactic acid is formed from glucose in muscle, the pathogenic bacteria split the glucose, converting it to lactic acid, thereby deriving energy. This reaction is, for practical purposes, the sole source of energy for certain pathogens such as streptococci, pneumococci, and some anaerobes. Other organisms can obtain further supplies of energy by breaking down the lactic acid to components with still smaller molecules, such as acetic acid, ethyl alcohol, and formic acid. Still others are able to oxidize the lactic acid and its decomposition products to carbonate, utilizing atmospheric oxygen in the process. Organisms capable of carrying out these reactions are obviously much better equipped and, in general, produce more luxuriant growth on a particular medium than those forced to use only the lactic acid fermentation.

Glucose, in addition to providing the chief source of energy for bacteria, is probably also the chief source of carbon, but this element may also be assimilated from proteins, their constituent aminoacids, alcohols, and organic acids of various types. The chief form in which nitrogen is assimilated by the pathogenic bacteria is as aminoacids. These they obtain either free or as a result of hydrolysis of the soluble digestion products of proteins, such as albumoses, peptones, and polypeptides which many types can attack by means of their proteolytic enzymes. It is now known that an aminoacid mixture can satisfy the nitrogenous requirements of the

pathogenic bacteria, and hence it has been found possible to grow many of them on purely synthetic media. Such media commonly require the addition of vitamins or similar substances, such as vitamin B<sub>1</sub>, and nicotinic acid amide to enable growth to take place.

It has been established that the line dividing the pathogens from those saprophytic bacteria which are able to utilize ammonium salts as their source of nitrogen is a narrow one. A pathogenic organism, such as *Bact. typhosum*, can be trained, by gradually withdrawing the aminoacids in the medium in which it is grown, to rely eventually upon ammonium salts as its sole source of nitrogen.

Hydrogen and oxygen are both assimilated with the organic compounds utilized during growth. Aerobic organisms both utilize and assimilate atmospheric oxygen, but the anaerobes are incapable either of utilizing or of assimilating gaseous oxygen. The explanation of this is uncertain, but it has been suggested that the obligatory anaerobes, when free oxygen is supplied, produce hydrogen peroxide which is inimical to them. Certain aerobes produce the same substance and also a catalase which breaks it down as rapidly as it is produced.

The many other elements such as sulphur, phosphorus, calcium, magnesium, sodium, and iron, which are almost invariable constituents of bacteria, are readily assimilated from the environment of the organism.

Each species of bacterium flourishes most abundantly within a certain restricted range of temperature, which varies from 0° to 5° C. in some marine species to 70° C. in the thermophilic bacteria of decaying manure and of hot springs. For each type there is a minimum, an optimum, and a maximum temperature of growth. In the case of many pathogenic microorganisms, the minimum is from 15° to 20°, the optimum about 37°, and the maximum 42° to 43° C. Although growth ceases at temperatures below the minimum and above the maximum, death does not necessarily occur, even with prolonged exposures to these temperatures. Extreme cold is especially well borne, and many bacteria which are pathogenic for man are capable

of surviving an exposure to  $-200^{\circ}\text{C}$ . for some hours. High temperatures are much more deadly, and an exposure of ten minutes to a temperature of  $60^{\circ}\text{C}$ ., in the moist state, is sufficient to kill most of the non-sporing pathogenic bacteria. When these are thoroughly dried, however, their resistance is greatly increased and even such a delicate organism as the pneumococcus, when dried over calcium chloride, may survive exposure to a temperature of  $115^{\circ}\text{C}$ . for half an hour. Spores are very much more resistant and may not be killed, when moist, at the temperature of boiling water for ten or more minutes: in the dry state, some can withstand several hours' exposure to a temperature of  $130^{\circ}$  to  $140^{\circ}\text{C}$ . In speaking of the thermal death point of bacteria it is essential to specify not only the temperature and the time of exposure, but also the material in which suspended and its reaction, as survival time is decreased in acid fluids and in those containing a large amount of salts. Light, particularly bright sunlight, and artificially produced ultra-violet rays are most injurious to bacteria; many species are very quickly killed by these agencies, and even some highly resistant spores are destroyed by sunlight in a few hours.

Bacteria derive their name from the fact that the earliest types examined were rod shaped. The term "bacterium" is now, however, applied to all these micro-organisms and bears no relation to their morphology. Five morphological classes of the lower bacteria are recognized—

1. Cocci.
2. Bacilli.
3. Vibrios.
4. Spirilla.
5. Spirochaetes.

Cocci are more or less spherical organisms. Among them the perfect sphere is uncommon and an oval bacterium is generally included in this group provided its greater axis is not more than double the length of its lesser. Their behaviour after fission enables them to be divided into sub-groups—

- (a) Diplococci.
- (b) Streptococci.

- (c) Staphylococci.
- (d) Tetrads.
- (e) Sarcinæ.

Every coccus, when fission occurs, is for a time a diplococcus. In some, however, the stage is only temporary, while, in the true Diplococci, it is more or less permanent. A time comes when each of a pair is about to divide, and then, usually, the two part and each becomes a pair of diplococci. In the Streptococci the nature of the cell envelope is such that, after

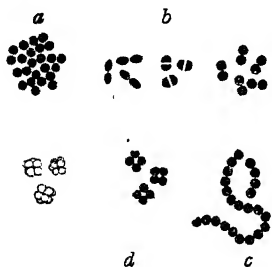


FIG. 2.—TYPES OF COCCI.

*a*, staphylococci; *b*, diplococci; *c*, streptococci; *d*, tetrads; *e*, sarcinæ.

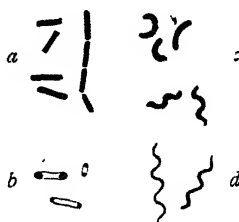


FIG. 3.—BACILLI, VIBRIOS, SPIRILLA, AND SPIROCHÆTES.

*a*, bacilli, single and in a chain; *b*, bacilli showing polar staining; *c*, vibrios and spirilla; *d*, spirochætes.

fission, the individuals do not readily part, and since, in these, fission proceeds in only one plane, a chain of cocci is produced. The Staphylococci divide (irregularly) in two planes, producing clusters like bunches of grapes. The Tetrads are a modification of staphylococci in which there is a tendency to form double pairs, that is, packets of four individuals. The Sarcinæ divide in three planes, producing groups of eight, resembling corded bales.

The Bacilli are cylindrical bacteria which may be either straight or slightly curved, long or short. Their long axis is at least twice their diameter. They always divide at right angles to their long axis, so the only possible classification, according to their mode of division, would be into those which occur singly, diplobacilli, and streptobacilli. These terms are sometimes used, but are not generally employed to dis-

tinguish different types. The bacilli are divided into a number of genera which will be dealt with later.

The rigid curved rods, the curve of which forms less than one complete turn, are called Vibrios. Each is usually provided with a single terminal flagellum.

Longer, rigid curved organisms, usually with several curves and with tufts of flagella at one or both poles, are Spirilla.

Spirochætes, the next group, are believed by some to be more correctly classed as protozoa than as bacteria. In them the number of curves is usually greater than in the spirilla, the organisms are flexible, and do not possess flagella.

Forms intermediate between the cocci and bacilli and between the bacilli and the spirilla are met with, and it is occasionally very difficult to say in which group a given organism should be put. In deciding the question regard should be paid only to the morphology of the bacterium when growing on suitable medium, for many micro-organisms, if the cultural conditions are unsuitable, appear abnormal in shape, the so-called involution forms. It must also be recognized that certain types have very different microscopic appearances when grown on different media; a bacillus, for example, may, under certain conditions, be so short as to resemble a coccus and, under others, may occur in long filaments.

The members of the next group to be considered differ in many ways from the preceding, and morphologically appear more nearly related to the fungi. They are characterized by the formation of filaments, which may be unbranched (*Leptothrix*) or may exhibit true branching (*Streptothrix*). Branching is not an exclusive property of these, the so-called "higher bacteria," as under certain conditions branching has been observed among such representatives of the lower bacteria as the tubercle bacillus and *C. diphtheriæ*.

Lastly, we have what is probably a large and heterogeneous group of living organisms which, until we can learn more about them, may be classed together as filterable viruses, filter-passing bacteria, or ultra-microscopic viruses. These

organisms are of such a minute size that they are either invisible or only barely visible with the highest magnifications of the microscope, and are able to pass through the pores of the coarser or, in some cases, of the finer filters capable of excluding the ordinary types of bacteria described above.

### **Safety in the Laboratory**

The following chapters describe the practical methods used in the study of bacteria, but, before proceeding to consider these, it is essential to warn the student of the dangerous nature of the materials with which he will work in the bacteriological laboratory and to point out to him the precautions which he should take both in his own interests and in the interests of others.

The bench should be covered with some impervious substance. If any material containing bacteria is spilled the bench should be immediately swabbed over with strong lysol solution (10 per cent.).

Scrupulous attention should always be paid to the sterilization, in the Bunsen flame or otherwise, of platinum wires, pipettes, or other pieces of apparatus which have come in contact with bacteria.

An enamel pail containing 2 per cent. lysol should be constantly beside the worker's chair to receive slides, Pasteur pipettes, and discarded cultures. In the case of plates, care should be taken to ensure the admission of the solution to the whole of the plate. Plugs should be removed from tubes before these are placed in the pail. A few hours' immersion in this disinfectant will kill the majority of bacteria, but, when material containing dangerous sporing organisms is dealt with, the whole pail should be autoclaved. A jar of the same solution should stand on the bench to receive contaminated graduated pipettes. When sterilization is complete the apparatus should be thoroughly washed in warm water, with soap and soda, and then in large quantities of plain water.

The hands should never, apart from accidents, come into actual contact with pathogenic material, but all cuts and

cracks about the fingers should be protected by rubber finger stalls. Rubber gloves should be worn when engaged in dangerous operations and always when performing post-mortem examinations on animals. Any wound received in the laboratory should at once be energetically disinfected with tincture of iodine or some other disinfectant such as Dettol.

When work is discontinued, the hands and arms should be bathed in 2 per cent. lysol, 15 per cent. Dettol, or other disinfectant, and well washed with soap and water.

The face, particularly the eyes, nostrils, and mouth, should be carefully protected from all contact with contaminated material. Mouth suction should not be applied directly to pipettes for measuring fluids containing bacteria. If teats cannot be employed suction should be applied through a rubber tube fixed to the end of the pipette. No food should be eaten in the laboratory. Gummed labels should never be moistened with the tongue.

No reasonable person attempts to prevent smoking in the laboratory, but it should not endanger the smoker. A pipe is safer than a cigarette. It should not be laid down so that the mouth-piece is in contact with the bench. A cigarette should never be laid on the bench. If the mouth part projects over the edge, the bench will be burned; if the cigarette is reversed, the mouth may become infected.

Students should remember that a number of careful and experienced bacteriologists have died of infections contracted in their laboratories.



## CHAPTER II

### MICROSCOPIC EXAMINATION OF BACTERIA

ON account of the extremely small size of bacteria, a microscope is essential for their study. To secure sufficient magnification a  $\frac{1}{12}$ -inch objective is a necessity and, in addition to this, we require a lens of somewhat lower power, about  $\frac{1}{6}$  of an inch. The  $\frac{1}{12}$ -inch lens is an "oil-immersion" lens since, in using it, a drop of cedar oil is placed on the object to be examined and the lens is immersed in the oil. An oil-immersion lens gives better illumination than a dry lens of the same power, for, when rays of light pass from object to lens through air, some are lost by refraction and others by reflection: since cedar oil has practically the same refractive index as glass, neither of these losses occurs with an oil-immersion lens. Such a lens also permits of a slightly greater working distance between the object and the lens and possesses the further advantage that greater detail can be made out than would be possible with a dry lens. Where cover slips are used with an oil-immersion lens they must be very thin (No. 1).

Further essentials in a microscope are a plane and concave mirror, a sub-stage condenser, which concentrates the light on the object, and an iris diaphragm. A movable stage and several eye-pieces or oculars of different powers greatly assist the student.

The source of light may be either the sky, electric lamp, incandescent gas-burner, or oil lamp. An artificial light is usually to be preferred as it is constant. The gas-filled, metal-filament electric bulbs (Fullolite, etc.) are probably the best source of illumination.

For the study of certain bacteria, particularly the spirochaetes, which are practically invisible in the living condition with ordinary illumination, a special form of condenser is employed. In this the rays of light are thrown on the object

very obliquely, so that none of them enter the objective. The bacteria and other objects are visible owing to the reflection of these rays from their surfaces and are seen in outline only. By the use of this apparatus very minute "ultra-microscopic" particles appear as bright points of light. This type of illumination is similar to that which renders visible the motes which are seen when a bright beam of light enters a dark room. For the use of a dark ground condenser it is essential to have a layer of oil between the condenser and the lower surface of the object glass, and also to have a very bright illuminant, such as an electric arc or a Pointolite lamp. With most types of dark ground condenser it is necessary to have a "funnel stop" which is dropped within the body of the objective, or else to use an objective with a built-in diaphragm, in order to cut off all direct rays of light.

In the use of the microscope with the ordinary condenser certain rules must be observed. For examining stained bacteria in films or sections, the oil-immersion lens is generally employed. With this the condenser and plane mirror are used, and the diaphragm should be widely opened unless the light is excessively bright; where an artificial light is employed intensity may be lessened by withdrawing the microscope to a greater distance. The mirror should be carefully adjusted and the condenser focussed so as to secure uniform illumination of the field. For studying living bacteria, a dry lens ( $\frac{1}{8}$ -inch) is preferable. With this the best illumination is obtained when the concave mirror is used without a condenser, and the diaphragm is nearly closed: if the light is too intense the bacteria cannot be seen.

By racking the microscope tube down carelessly it is easy, not only to ruin the preparation but also, a much more important thing, to dislocate or fracture the front lens of the objective. To avoid this catastrophe the following rules should never be departed from in focussing the microscope. The coarse adjustment should be used to lower the objective to within its working distance from the object. This is done with the eye actually observing the object and objective almost on a level with the former, and not stationed at the

ocular. Where the oil-immersion lens is used it should be lowered cautiously into the drop of oil, and then a little further, so that the drop is seen to be flattened out ; it must never come in contact with the cover glass. The eye is now applied to the ocular and the mirror adjusted to give good illumination. The coarse adjustment is worked so as to elevate the objective very slowly until the object is seen. If it is passed unobserved, it is necessary to repeat the operation from the beginning. The objective should never be racked down with the eye at the ocular as this may ruin the lens owing to its coming in contact with the slide. Once the object has been found, fine focussing is done with the fine adjustment, the mirror is arranged and the condenser focussed so as to get critical illumination. The fundamentals are : (1) Never rack down unless the objective is under actual observation ; (2) always " find " the image of the object by racking upwards with the coarse adjustment, never downwards ; (3) never use the fine adjustment to find the image, only to get the best possible focus.

In using the microscope, both eyes should be kept open, as this causes less strain than when one is closed. At first the two images will cause trouble, but after a time one becomes accustomed to ignoring what is seen with the eye not applied to the microscope, especially if the bench is dark in colour and not too brightly illuminated.

Bacteria may be observed microscopically either unstained in fluids, or stained in films or sections. The method of examining living or dead bacteria, unstained, will first be described. For this purpose we may make a hanging-drop preparation, using either a hollow ground slide or an ordinary slide with a glass, vulcanite, or metal ring about 2.0 cms. in diameter by 0.3 cm. high. In the latter case, the ring is held by a forceps and dipped in melted vaseline, when it is dropped on the centre of a slide. The hollow ground slide is prepared by running melted vaseline in a ring round the hollow. A drop of the broth culture, or other fluid containing the bacteria, is placed in the centre of a cover slip and the prepared slide lowered over it until contact is made between the vaselined

surface and the cover slip. The slide may now be inverted and the cover slip gently adjusted, so that the drop hangs suspended in a chamber sealed with the vaseline: this prevents the drop from drying. An alternative method is to place a drop of the fluid on a slide and to drop a cover slip on it. The drop should be neither too large, in which case some of it will flow out beyond the cover slip, nor too small, for, if it is, bubbles will form. If the preparation has to be kept for some time it may be sealed, so preventing evaporation, by running around the edge of the cover slip a thin glass rod dipped in melted paraffin wax. At

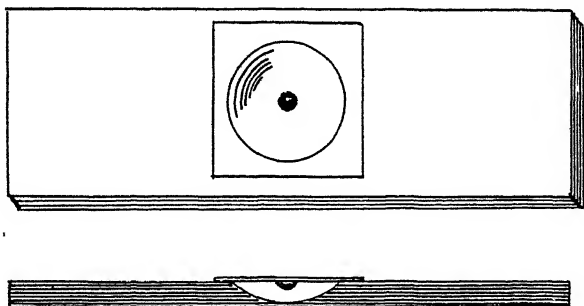


FIG. 4—Hanging Drop Preparation.

first it will be found difficult to see the bacteria on account of their transparency. Artificial light, no condenser, concave mirror, and a diaphragm almost closed will assist. It may help the beginner to focus the bacteria if a mark is made with a grease pencil at one side of the lower surface of the cover slip before the preparation is mounted. If this is brought sharply into focus and the slide then moved, so that the drop is below the objective, the bacteria themselves should at once be visible. It is necessary to determine whether these are motile or not. True motility must be distinguished both from Brownian movement, which is due to molecular bombardment, and is seen in minute, lifeless particles, and also from the flowing of the bacteria owing to currents set up by uneven heating. The best test is to observe carefully two bacteria lying close together and sharply in focus at

the same time. If these move in different directions, they exhibit true motility. It is necessary to guard against wrong conclusions which may arise from unusual activity of non-motile organisms or from sluggishness of those normally motile. Excessive Brownian movement or sudden changes in surface tension, such as occur when bacteria from a solid medium are suspended in water, may simulate motility, and an unfavourable medium, too low a temperature, or the presence of oxygen (in the case of some of the anaerobes) may cause a motile organism to become non-motile. All organisms which, when suitably stained, show flagella are motile in favourable circumstances, but the absence of flagella is not, in all cases, equivalent to the absence of the powers of locomotion. By continuous observation of a bacterium in suitable medium and at a suitable temperature it is possible to discover its method of multiplication.

As very little can be discovered concerning bacteria in unstained preparations, apart from the question of motility and of reproduction, the methods of staining bacteria are of great importance. For satisfactory preparations clean slides, free from grease, are essential, as the least trace of any oily substance will prevent a drop of water spreading in a thin, even film. The slides may be treated with a solution of potassium bichromate in dilute sulphuric acid, followed by washing in water and drying, or may be heated strongly in the Bunsen flame. In the case of a fluid, whether culture medium, urine, cerebro-spinal fluid, or other body fluid, it is only necessary to place a drop of this in the centre of the slide and spread it slightly with the platinum loop. The bacteria and cells in a fluid, such as urine, may be concentrated by centrifuging, films being made from the deposit. Films of pus may be spread with a platinum loop. A very thin, even film is, however, desirable, and better preparations may be secured by dipping a cotton wool swab in the pus, expressing the excess against the wall of the tube and then "dabbing" the swab on the slide several times, each time on a fresh surface. If the pus is very thick, or contains small nodules of caseous material, the same procedure may be adopted as is

used with sputum. A fragment is placed on the slide and another slide applied to this; the two are tightly pressed together, rubbed over one another, so breaking down the nodules, and are then drawn apart. Blood films are made by placing a small drop of blood towards one end of a slide. The

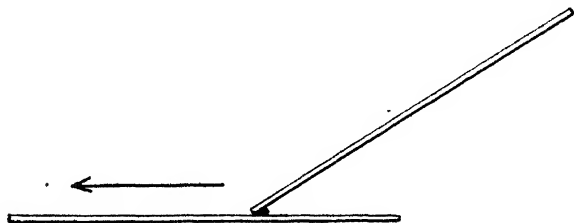


FIG. 5.—METHOD OF SPREADING BLOOD FILM.

edge of another slide is moved along the surface of the first until it comes in contact with the drop, when the blood spreads out between the two. The second slide is held at an angle of about  $30^\circ$  with the first and pushed slowly over its surface. The blood is drawn along with the upper slide and produces a thin film on the surface of the lower.

To make a film from a culture on solid medium, a drop of water is placed on a slide and the platinum wire allowed to touch the culture, a small portion of which adheres to it. The wire is then rubbed in the drop which is spread out to form a film. Great care must be taken to avoid adding too much culture to the water as the chief error likely to occur is to make the suspension far too thick: it should be but faintly opalescent. Beginners sometimes find it hard to understand why tap water, containing bacteria, may be used in preparing films, but must not be added to cultures. Let us suppose that the water contains 100 bacteria per c.c. A platinum loopful added to a tube of broth would probably contain two bacteria which might grow in the broth when it is incubated and so contaminate the culture. But if a loopful were used for the preparation of a film, the latter would probably measure about 4 sq. cms. The field seen with an average oil immersion lens measures about 0.0002 sq. cm., that is  $\frac{1}{20000}$  of the area of the film. From this it follows that one of the two water

bacteria introduced would be seen in the examination of something like 10,000 fields. The chances against any bacteria present in the water being seen in the microscopic examination of a film are, then, very remote.

Films, however prepared, must be fixed. They may be fixed wet by dipping into a suitable fixing solution, of which one of the simplest and best consists of one part of formalin and nine parts of absolute alcohol. After three minutes in this the film is well washed in spirit. Wet fixation is only employed when the finer points of cytology are under examination. As a routine method the films are first dried and then fixed. Drying is best done at air or body temperature (in an incubator), protected from dust. If the amount of fluid taken is large the film will take a long time to dry, and there is a great temptation to hasten drying by heating, which is one of the commonest causes of the unsatisfactory films frequently made by students. The most that can be allowed is to wave the film about, high above the Bunsen, where the fingers are not uncomfortably heated.

When the film is dry it is fixed, so that the bacteria and cells are made to adhere firmly to the slide. This may be accomplished by the use of absolute alcohol or a mixture of equal parts of absolute alcohol and ether, but more commonly the film is fixed by passing it three or four times slowly through the Bunsen flame, film side upwards. Overheating will be avoided if, during heating, the slide is held between the finger and thumb, and if, after heating, the temperature of the under surface of the slide is tested by holding it against the sensitive part of the back of the hand which lies between the base of the thumb and forefinger. When cool, and not before, it is ready for staining.

The various staining methods will be given below, but the method of treating the film, after staining, may be here described. Usually staining is concluded by washing with water. The slide is laid between several sheets of fluffless blotting or filter paper on a smooth table, and the covering sheets are pressed into contact with the film with the edge of the palm, any rubbing of the paper on the film being

avoided. The film is then allowed to dry completely by waving it above the Bunsen flame. It may be treated with a drop of xylol, mounted in Canada balsam, and covered with a cover slip, but unless permanent preparations are required, the film, after drying, is usually examined unmounted, a drop of cedar oil being applied and the oil-immersion lens used. If it is desired to keep such a film, the oil may be removed with xylol and the film stored, after drying, protected from dust.

Sections to be stained for bacteria must be thin (about  $5\mu$ ) and, for this reason, paraffin sections are preferable to those cut with the freezing microtome. The methods of cutting and mounting such sections are described in textbooks of Histology and Pathology. To stain a paraffin section it is necessary to remove the paraffin completely with xylol and this in turn with alcohol. After washing with water the section may be stained as described for films, but it is advisable to increase the time given to each operation, especially decolorization, where that is necessary. As the usual histological process of dehydration with absolute alcohol is very prone to remove some of the stain, excess of water may be removed by careful blotting and dehydration of sections may be accomplished with aniline oil, aniline-xylol, and then xylol. A less perfect, but sufficiently good method for most purposes, is to blot the section, allow to dry in air and clear in xylol. It is then mounted with a cover slip (No. 1) in Canada balsam.

Since stains are very liable to soil the bench, staining should be done on a rack, over a sink. Such a rack consists of two parallel glass or metal rods arranged so as to be absolutely horizontal and about two inches apart; on these the slides rest during staining, and if heat is required it may be conveniently applied with a small torch made by moistening a cotton swab on a wire with spirit. Soiling the fingers is avoided by using Cornet's forceps for lifting the slide when desired.

Of the many solutions used for the staining of bacteria, by far the greatest number contain one or other of the basic aniline dyes dissolved in water which may, or may not, contain small amounts of alcohol, alkalies, or acids. The



weak aqueous solutions actually used for staining do not usually keep well, and it is, therefore, convenient to make saturated solutions of the dye in water or, more often, in alcohol, since these are stable. The selection of a suitable staining method for each case depends on the experience of the operator. Some stains, such as methylene blue or thionin blue, are easy to use, since they do not readily overstain. Dilute carbol-fuchsin, gentian violet, or methyl violet act very rapidly and tend to overstain the preparation, and must, therefore, be used with care. In some methods two stains of contrasting colours are used and much information may be derived from the action of these. Certain bacteria stain only with great difficulty, and for successful results it is necessary to intensify the action of the stain by heating, by the addition of alkali, or carbolic acid, or by the use of a mordant such as tannic acid.

### Simple Stains

#### Löffler's Alkaline Methylene-blue.

1 per cent. aqueous potassium hydrate	1.0 c.c.
Water	99.0 c.cs.
Saturated alcoholic methylene-blue	30.0 c.cs.

Films are stained for from five to ten minutes, washed with water and dried. Sections are stained for fifteen to thirty minutes. The differentiation of the bacteria from the cells may be improved by decolorizing for a few seconds in  $\frac{1}{2}$  per cent. aqueous acetic acid, which removes some of the colour from the cells but does not affect the bacteria. The sections are then washed and dried.

#### Carbol-Thionin blue.

Thionin blue	1.0 gm.
$2\frac{1}{2}$ per cent. phenol in water	100.0 c.cs.

This is a stock solution. For use dilute 1 in 4 with water and filter. This is a particularly useful stain for sections. Stain for ten to fifteen minutes, differentiate in 1-1,000 acetic acid, wash and dry. Bacteria usually take a more

purple tint than the tissue cells, which are of an almost pure blue colour.

### **Methyl Violet.**

Methyl violet	.	.	.	.	.	0.5 gm.
Water	.	.	.	.	.	100.0 c.cs.

This is an intense stain which will stain bacteria in films in thirty seconds or less. It is not frequently used as a simple stain, but as a part of Gram's method. Gentian violet may be used in much the same way and, in combination with aniline oil or phenol, was formerly used in Gram's method, but both aniline-gentian violet and carbol-gentian violet are unstable and must be freshly prepared every few days.

### **Carbol-fuchsin.**

Basic fuchsin	.	.	.	.	.	1.0 gm.
Absolute alcohol	.	.	.	.	.	10.0 c.cs.
5 per cent. aqueous phenol	.	.	.	.	.	100.0 c.cs.

This stain is too powerful for ordinary bacteria as films are overstained with it in a few seconds. Dilute carbol-fuchsin (1 part of the strong stain to 19 of water) is a simple and useful stain for films ( $\frac{1}{2}$  to 1 minute), but is liable to obscure detail if cells are present.

## **Double Staining**

**Gram's Method.**—Bacteria can be divided into two great groups, the Gram positive and the Gram negative, and to ascertain to which class an unknown organism belongs is usually one of the first steps in its identification. In Gram's method the organisms are first stained with crystal, gentian or methyl violet, and then treated with an iodine solution. When washed with alcohol, Gram positive bacteria are unaffected, but those which are Gram negative, as well as the great majority of tissue cells, lose the violet colour. A counter-stain is used in order to stain Gram negative organisms and any cells present. The method is one of the most difficult of those commonly employed, but the technique must be thoroughly mastered before any advances can be made in

bacteriology. The difficulty most usually encountered is in the decolorization. Most bacteria are definitely positive or negative, but, with a few, the result may be doubtful since they appear positive if decolorization is curtailed and negative if it is prolonged. The real difficulty here is not in the staining, but in the preparation of the film. If this is uneven, thick in one part and thin in another, a good result is practically impossible, since, if the thin parts are correctly treated with alcohol, the thick portions are not sufficiently decolorized, while, if attention is paid to these, the thin parts will be over-decolorized. Most of the positive bacteria can be made to appear Gram negative by prolonged treatment with alcohol. Old or degenerated Gram positive organisms, or those grown on unsuitable media, are frequently found to be Gram negative. There are many modifications of the original Gram's method, in which aniline-gentian violet, an unstable stain which only keeps for a few days, and a weaker iodine solution were employed, but the one here described, that of Jensen, is probably the simplest and most reliable.

- (a) Crystal violet. . . . . 0.5 gm.  
 Water . . . . . 100.0 c.cs.

Stain for  $\frac{1}{2}$  minute.

- (b) Iodine . . . . . 1.0 gm.  
 Potassium iodide . . . . . 2.0 gms.  
 Water . . . . . 100.0 c.cs.

The violet stain is rapidly washed off with water, the film rinsed with the iodine solution and then covered with that solution, which is left on for one minute.

(c) The iodine solution is poured off and the slide shaken to remove as much of it as possible. The film is then rapidly flooded with alcohol, the flooding being twice repeated to remove all traces of iodine without delay. It is then covered with alcohol and rocked, as is a photographic plate during development. The alcohol takes up some of the violet colour and when this occurs it is poured off and replaced with fresh. The whole washing process is repeated several times until fresh alcohol removes no more violet from the film. Only

practical experience will teach how much washing with alcohol is correct. Films prepared from cultures on solid media may require only a few seconds, films of pus one or two minutes.

(d) The film is then counter-stained. Most commonly dilute carbol-fuchsin is used, as described above, but the contrast between the colour given by this, if allowed to act for too long a time, and the violet is not always very marked. Some bacteriologists prefer to counter-stain for half a minute with a 1/1000 solution of neutral red, acidified with weak acetic acid.

An alternative counter-stain is prepared by diluting a saturated watery solution of Bismarck brown with an equal quantity of water and filtering. Films are stained in this for about five minutes. It is chiefly of use when attention is directed towards the Gram positive organisms, as those which are Gram negative and cells are only slightly stained.

(e) Finally the film is washed with water, blotted and dried. In connection with this stain a few words must be added. The violet must be that sold as "crystal violet pulv. standardized" by Hollborn, which is identical with that formerly produced by Grübler under the name of "methyl violet 6B." We have found no other stain so satisfactory and some so-called methyl violets are quite useless.

Special modifications of Gram's method are used for sections, but steps (a) and (b) above may be applied successfully, allowing double the indicated time. Decolorization (c) will take much longer than for films. It is advisable, after decolorization, to treat the section with acetone for a few seconds as this removes crystals which occasionally prove troublesome. Counter-staining may be performed with 1 per cent. alcoholic eosin, for two or three seconds: dilute carbol-fuchsin should not be used.

Excellent practice is afforded in the technique of Gram's method by making films of a mixture of a staphylococcus and *Bact. coli*. After some time it will be found possible to produce a film in which all the cocci are violet and all the bacilli red or brown, depending on the counter-stain used. In examining preparations stained with Gram's method it is of importance that the diaphragm of the microscope should be widely open

as the distinction between Gram positive and negative bacteria is more apparent with good lighting.

**Neisser's Stain.**—This stain is used to demonstrate the granules of *C. diphtheriæ* which appear of a blue colour, the rest of the body of the bacillus being stained brown. The culture used should be taken from a solidified serum medium, as bacilli from agar or tellurite media, or those in the membrane do not show the blue granules so well.

- (a) Methylene blue . . . . . 1·0 gm.  
(dissolve in 20 c.cs. of 96 per cent.  
alcohol and add

Water . . . . . 950·0 c.cs.

Glacial acetic acid . . . . . 50·0 c.cs.

Stain the film for about three minutes and wash rapidly in water. Then counter-stain with

- (b) Bismarck brown . . . . . 2·0 gms.

Water . . . . . 1,000·0 c.cs.

Stain for three minutes, wash rapidly in water, blot and dry.

#### **Pugh's Stain.**

5 per cent. alcoholic solution of toluidin blue 1·0 c.c.

2 per cent. aqueous acetic acid . . . . . 50·0 c.cs.

This is also used for demonstrating the granules in diphtheria bacilli. These appear blue, the bodies being almost unstained. Make the film on a slide, dry and fix in alcohol or by heat. A drop of the stain is placed on the slide and a cover slip inverted on it. The preparation may be examined immediately with the oil-immersion lens. These preparations are, of course, not permanent.

#### **Ziehl-Neelsen Method for Tubercle Bacilli.**

- (a) Basic fuchsin . . . . . 1·0 gm.

Absolute alcohol . . . . . 10·0 c.cs.

5 per cent. phenol in water . . . . . 100·0 c.cs.

The film is made and fixed in the ordinary way. It is covered with the stain and heat applied below the slide either with a small flame of the Bunsen, a spirit lamp, or spirit torch. The heating should be sufficient to cause steam to rise, but boiling the stain must be avoided. Staining should last for

five minutes, heat being applied occasionally. Care must be taken to prevent the stain drying on the slide, fresh stain being added if necessary.

(b) Wash well in water.

(c) Immerse the slide in 20 per cent. sulphuric acid in water. It is convenient to have a bath containing this fluid into which the slide may be dropped. In the acid the pink colour changes to yellow or brown.

(d) Wash in water. The pink colour may return. If so steps (c) and (d) are repeated as many times as necessary, until the film has not more than a faint pink tinge.

(e) Wash in alcohol one minute.

(f) Wash in water.

(g) Counter-stain with Löffler's methylene blue for one half-minute.

(h) Wash with water, blot and dry. Sections are treated in the same way as films. A more rapid method is to replace steps (c), (d), and (e) by using, as a decolorizer, acid-alcohol (spirit containing 3 per cent. hydrochloric acid). After about one minute in this, rinse in water, and, if too pink, replace in the acid-alcohol and repeat if necessary. Steps (f), (g), and (h) are carried out as above. Some prefer to use Bismarck brown, saturated aqueous picric acid, or a solution of brilliant green as a counter-stain in place of methylene blue, which, if applied for too long, may obscure bacilli. The method may be used for other acid-fast bacilli as well as the tubercle bacillus, but, since many of these are acid- but not alcohol-fast, step (e) (alcohol) should be omitted and acid-alcohol should not be used. The step is important when tubercle bacilli are being looked for, as the alcohol decolorizes many of the bacilli likely to cause confusion. In a properly stained preparation the acid-fast bacilli appear bright red, and other bacteria, as well as cells, the colour of the counter-stain used.

### Methods for Demonstrating Spirochætes

**Levaditi's Method.**—For tissues it is most satisfactory to employ the method of Levaditi, in which the spirochætes are impregnated with metallic silver. The tissue is then embedded

in paraffin and sections cut. These, when fixed to slides, merely require to be freed from paraffin with xylol and mounted in balsam. The spirochætes appear black and the tissues brown.

**Fontana's Method.**—For demonstrating spirochætes in films the most satisfactory method is Fontana's. The film is fixed after drying, without heat, in

(a) Acetic acid . . . . .	1.0 c.c.
Formalin . . . . .	20.0 c.c.s.
Water . . . . .	100.0 c.c.s.

The film is treated with the solution, which is changed two or three times, for about one minute.

(b) Wash well in water.

(c) Tannic acid . . . . . 5.0 gms.  
1 per cent. phenol in water . . . . . 100.0 c.c.s.

Cover film with this and heat till steam rises, but do not boil. Treat thus for half a minute.

(d) Wash well in water.

(e) Silver solution is prepared thus:—To a 1 per cent. solution of silver nitrate, add, drop by drop, 10 per cent. ammonia solution when a turbidity appears. Continue adding the ammonia, with constant shaking, until the solution becomes almost clear. The film is covered with the solution so prepared and gentle heat is applied for half a minute.

(f) Wash well, blot and dry.

The spirochætes are stained dark brown or black.

**Indian Ink Method.**—Spirochætes can be demonstrated (unstained) by the Indian ink method. Some fine Indian ink, of good quality, is diluted with an equal quantity of water, and a loopful of this is well mixed with a loopful of the material containing the spirochætes. A thin film is made and allowed to dry, when it may be examined. Spirochætes and other bacteria stand out well, unstained, against a brown background of the ink.

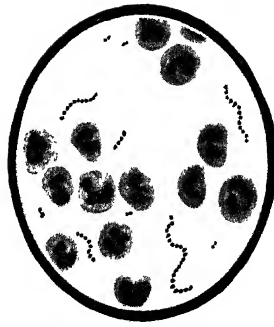
### Compound Stains

**Leishman's Stain.**—This is used chiefly for staining films of blood, and most of the blood parasites, such as those of

# PLATE I.



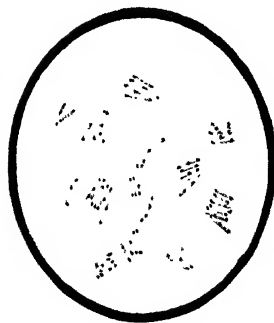
1.



2.



3.



4.

Bacteria as seen with the oil-immersion lens.

1. Gonococci in pus from urethra.

2. Streptococci in pus.

3. Tubercle bacilli in sputum.

4. *B. diphtheriae* (Neisser's stain).

*To face page 26*





malaria, the trypanosomes and spirochaetes, are well seen in films stained with it. It is most convenient to buy the dry prepared stain and to dissolve this in pure, acetone-free, methyl alcohol to the extent of 0.15 per cent. The film of blood is dried, without heating, and is fixed by covering it with the stain. This is left on for about one minute, when about four times the quantity of distilled water is added. The stain and added water must be thoroughly mixed, very conveniently, by drawing up into a capillary pipette and expelling two or three times. The dilute stain is left on for five to ten minutes, the slide is well washed with water, blotted and dried.

**Pappenheim's Methyl Green-Pyronin Stain.**

Methyl green	.	.	.	0.15 gm.
Pyronin	.	.	.	0.50 gm.
95 per cent. alcohol	.	.	.	5.0 c.cs.
Glycerol	.	.	.	20.0 c.cs.
2 per cent. phenol in water	.	.	.	75.0 c.cs.

This is a very satisfactory stain for demonstrating bacteria in sections and films of pus. Bacteria are stained pink, while the great majority of cells appear a faint blue. Sections are stained for about fifteen minutes. The stain is washed off with water and the section decolorized with 70 per cent. alcohol in water till it appears blue. It is then dried, mounted, and examined. Films are stained for five minutes and are then washed with water, blotted and dried.

**Special Stains**

**Möller's Method for Staining Spores.**—Films are dried and fixed by heat.

(a) Stain with carbol-fuchsin (strong) for ten minutes, heating as in the Ziehl-Neelsen method.

(b) Wash in water.

(c) Decolorize in 1 per cent. sulphuric acid or in spirit (not both) for 30 seconds.

(d) Wash in water.

(e) Counter-stain with methylene blue for 2 minutes.

(f) Wash in water.

(g) Blot, dry, and examine.

Spores of different bacteria vary somewhat in the ease with which they stain and decolorize by this method and, in order to get the best results, it may be necessary to make a few experiments, particularly with regard to the time devoted to step (c). Spores are stained red and the bodies of the bacilli blue.

**Capsule Stain—Method of Hiss.**—This succeeds best in films freshly made from the body. With pneumococci in the blood of an infected mouse, for example, very good results are obtained. Films from cultures should be made in a drop of dilute serum instead of water. Films are dried and fixed by heat.

(a) Crystal-violet solution used in Gram's method. Cover film with the stain and heat until it steams, stain for half a minute

(b) Wash off the stain with 20 per cent. aqueous copper sulphate.

(c) Blot and dry. Do not wash with water. A faint bluish-purple capsule is seen surrounding the darkly stained bacterium.

**Indian Ink method of demonstrating capsules.**—Films (preferably from a culture on solid medium) are made and dried in the usual way. A thin film of Indian ink (Higgins's water-proof Indian ink is satisfactory) is spread, in the same way as a blood film, over the surface of the bacterial film. This is dried with gentle heat. The preparation is stained with hot methyl violet (or crystal violet), washed with water, treated with Gram's Iodine for one minute, blotted and dried. The bacteria are stained black and are seen to be surrounded by unstained halos which stand out against the dark background. These halos are the capsules.

## CHAPTER III

### STERILIZATION

IN the cultivation of bacteria we have two cardinal points requiring attention—the preparation of food material and the exclusion of extraneous organisms. Of these the latter is of such fundamental importance that it will be dealt with first. Sterilization is the name given to the process by which bacteria are destroyed. It may be accomplished in a variety of ways, each of which has its uses in the laboratory.

#### **Sterilization by Heat**

This is a simple and satisfactory method, which is capable of many modifications. Useless articles, contaminated paper or dressings, bodies of infected animals and the like, are rendered harmless by burning. Small pieces of apparatus, particularly of metal such as platinum wires and forceps, are satisfactorily sterilized by heating to dull red heat in the flame of a Bunsen burner. Small pieces of glass, such as slides, capillary pipettes, and thin glass rods may also be rendered sterile by heating in the flame; but the temperature must not be sufficient to fuse them. When the flame begins to become yellow the glass is certainly sterile, as this indicates that fusion is about to commence.

Larger articles of glass—test tubes, flasks, Petri dishes—as well as cotton wool and paper, cannot be sterilized in this way. For them the “Hot air sterilizer” is used. This is a double-walled metal chamber which is heated below by a group of Bunsen burners, the hot air rising between the inner and outer walls. A thermometer for reading the temperature of the interior is essential. The temperature and the time of heating are both important, as the process must be continued long enough to kill all spores. It has been found that 160° C. for one hour is ample for this purpose; but an exposure at 140° C.

for three hours is preferable, as this causes less destruction of organic material such as paper or cotton wool. Glass vessels must be put into the chamber before it is heated, and left to cool in it after the gas is turned off, as any rapid change in temperature is very liable to crack glass, through sudden expansion or contraction.

Moist heat is a much more efficient agent in killing bacteria than is dry heat. It can be applied by boiling, as is done for syringes, surgical instruments and other small pieces of apparatus. Five minutes' boiling will kill all non-sporing

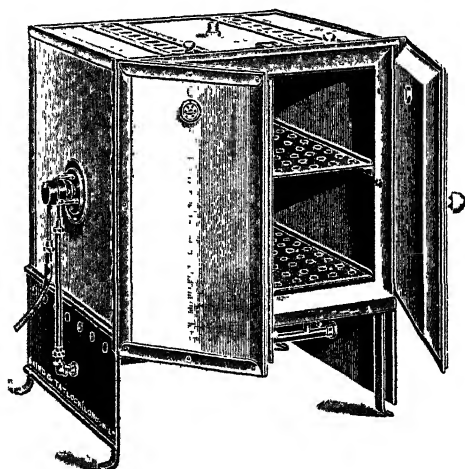


FIG. 6.—HOT AIR STERILIZER.  
(Messrs. Baird and Tallock (London), Ltd.)

bacteria and the majority of spores, but for complete destruction of all bacteria one hour or more is required.

For larger apparatus a "steamer" is used. This, in its simplest form, is merely a large metal vessel provided with a lid and containing some water in the bottom. This water is boiled by means of gas burners, and from it the steam rises and fills the vessel, escaping through a small opening near the top. The newer steam sterilizers, such as the Arnold, are an improvement on the old since steam can be raised in them

in a few minutes. Flasks or other vessels rest on a perforated shelf situated above the level of the water. The steamer is used for sterilization of media or other material. An exposure to a temperature of  $100^{\circ}\text{C}$ . for a few minutes is sufficient to kill all non-sporing bacteria; but one must remember that some time is needed for steam to heat the fluid contained in a large flask to its own temperature. For this reason vessels of media are generally left in the steamer for twenty to thirty minutes after the water has commenced to boil. Spores are not all destroyed by steaming but, by the method of "intermittent" or "fractional" sterilization, they may usually be killed. This consists in steaming for half an hour on each of three days, the vessel being kept at air temperature between each exposure. The rationale is that the first steaming kills all vegetative forms. In the interval between this and the next exposure any spores present assume the vegetative form, and these are killed at the second steaming. The third heating is an additional precaution. The same intermittent method may be applied at lower temperatures than that of steam, *e.g.*  $60^{\circ}\text{C}$ . In this case the number of exposures is usually increased to five or six. It happens, occasionally, that the method may fail, owing to the presence of spores of anaerobic bacteria, which do not find themselves, in the intervals, in favourable circumstances for the development of their vegetative forms.

Although rarely employed in the laboratory, some mention



FIG. 7.—STEAM STERILIZER  
(ARNOLD PATTERN).  
(Messrs Gallenkamp.)

must be made of pasteurisation. This process, which is chiefly used for the treatment of milk, consists in keeping the fluid at a temperature of  $63^{\circ}$  to  $65^{\circ}\text{C}$ . for half an hour. While not sufficient to sterilize, this is sufficient to kill most of the pathogenic bacteria likely to occur in milk.

The next method is by the use of the autoclave, which is a very strong boiler with a lid which can be hermetically sealed. The autoclave, which must be provided with a pressure-gauge, a valve for releasing pressure and a safety valve, contains water in the bottom. The vessels are placed on a perforated shelf and the lid screwed down. Heat is applied by gas burners, and when the water boils steam emerges from the valve. It must be allowed to flow for some time in order to expel all air, for the pressure-gauge acts also as a thermometer, but its readings are unreliable unless all air has been expelled. After a few minutes the valve is closed and the pressure in the interior, as indicated by the gauge, rises. The greater the pressure the higher is the temperature; 10 lbs. pressure

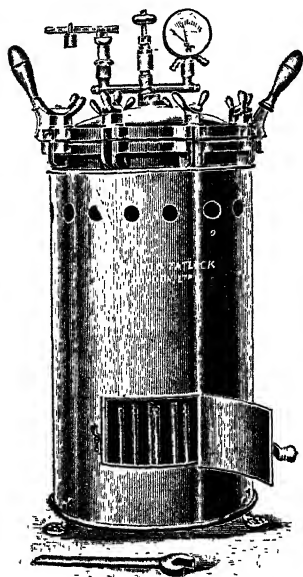


FIG. 8.—AUTOCLOVE.  
(Messrs. Baird and Tatlock  
(London), Ltd.)

per square inch is equivalent to  $115^{\circ}\text{C}$ . An exposure to this temperature for fifteen minutes will kill practically all known forms of life. This temperature should not be exceeded, owing to the harmful effect of high temperature on many forms of medium. After the requisite time has elapsed, the gas is turned off and the apparatus allowed to cool. The valve must not be opened until the internal pressure is the same as, or below, that of the outside air, as otherwise

the fluids will boil violently and expel the plugs from the tubes or flasks. The valve should always be opened before the lid is unscrewed. Before use it is essential to see that the apparatus contains sufficient water, both to avoid accident and also to prevent the steam from being superheated, since saturated steam is much superior in disinfecting power to superheated steam. Autoclaving is the quickest and most certain method for the sterilization of media; but it cannot be used for all media, since some varieties are injured at temperatures above 100° C. In general, it may be said that it is well to employ the lowest temperature for the shortest time compatible with complete sterilization in the preparation of culture media. Over-heating never does good, but is frequently harmful.

### **Sterilization by Chemical Means**

Another method of sterilization is by the use of chemical disinfectants. This has a small sphere in the preparation of media, but is extensively used in the laboratory for the disinfection of contaminated material, small pieces of apparatus and the hands. Biniodide of mercury ( $\frac{1}{1000}$ ), phenol (5 per cent.), formalin (10 to 20 per cent.), all have their uses, but probably lysol (2 per cent.) is of the greatest utility.

A volatile antiseptic, such as chloroform, is sometimes used to preserve culture media until the latter are required, when the chloroform may be expelled by heat.

### **Other Methods of Sterilization**

The passage of an electric current has been used as a method of sterilization in some cases, but it is probable that the effect is due either to heat or to the liberation of chemical substances (such as chlorine, when sodium chloride is present in solution) and not to electricity *per se*.

The foregoing methods all have for their purpose the killing of bacteria. The remaining method of sterilization is one by which bacteria, whether dead or alive, are removed from fluids



by filtration. If a liquid is passed through a porous material, none of the pores of which are larger than the smallest bacterium present, it will, after its passage, be completely free of bacteria. This process of sterilization is considered in greater detail in Chapter VI.

### Maintenance of Sterility

Up to the present we have considered only the methods of sterilization ; the next question is how we can prevent bacteria from the air entering apparatus or fluids which are sterile. By far the most useful method is by plugging the mouths of tubes and flasks with non-absorbent cotton wool. Provided the cotton is dry, no bacteria can penetrate through its

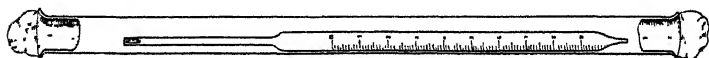


FIG. 9.—STERILE GRADUATED PIPETTE.

interstices. If, however, it is damp, certain micro-organisms, particularly moulds, can grow through it and contaminate the material within. Test tubes and flasks prepared to contain media are plugged fairly tightly with cotton wool and heated for three or four hours at  $140^{\circ}\text{C}$ . in the hot air sterilizer. Petri dishes are wrapped in paper and sterilized in the same way.

The mouthpieces of graduated pipettes should be plugged with cotton wool. The pipettes are placed in long thick-walled glass tubes, both ends of which are plugged with cotton wool. These are sterilized in a hot air oven. The contained pipettes remain sterile until required.

## CHAPTER IV

### THE PREPARATION OF CULTURE MEDIA

THE materials used for the preparation of a culture medium suitable for the growth of a bacterium depend largely on the natural habitat of that particular organism. Bacteria which are parasites on plants thrive best on media containing extracts of those plants, and those pathogenic for fish on media composed largely of the muscles of fish.

The bacteria with which we are chiefly concerned are those pathogenic to man and, for their cultivation, we must supply materials similar to those which they find available in the human body. These organisms obtain the energy necessary for their metabolic processes and other activities mainly by splitting fermentable carbohydrates and, to a less extent, by splitting and oxidizing such salts as lactates. From the same sources they obtain the carbon required to build up their protoplasm. Nitrogen is obtained from aminoacids, some of which are indispensable and others desirable. Hydrogen and oxygen are supplied mainly by carbohydrates and, to a less extent, by aminoacids. It is doubtful if any of the gaseous oxygen essential for the growth of aerobes is actually assimilated: it is used for the oxidation of food-stuffs, so supplying energy. Sodium, potassium, sulphur, and phosphorus are essential elements, sufficient of which are present in the tissues and body fluids. Vitamins, such as B<sub>1</sub>, which are required by some bacteria, and other substances, minute amounts of which are essential for the growth of certain pathogens (*e.g.* the iron containing derivative of haemoglobin for *H. influenzae*), are available in the body.

An extract of fresh meat supplies practically all the essentials mentioned but, although it will permit the growth of the majority of pathogens from even the smallest inocula-

tion, a denser growth is obtained if peptone is added to it. This both increases the amount of aminoacids available and acts as a buffer.

A broth prepared from an extract of meat and peptone contains hexose derived from the glycogen of the muscle. This is the chief source of energy utilized by bacteria. It also contains pentoses which can be used by some bacteria to supply energy. Since the total amount of carbohydrate present in such a broth is small, it is often an advantage to supplement it by the addition of a small amount of glucose. This is particularly useful when streptococci or pneumococci are grown in it.

A good medium should not merely include desirable materials, but should also exclude undesirable substances of which one of the commonest and most prejudicial is copper. This metal is, or until very recently was, almost a constant contaminant of commercial peptones. Care should, therefore, be taken either to use a copper-free peptone or to prepare media in such a way as to remove it. In the preparation of broth, as described below, any copper present combines with the meat and is removed with it by filtration. It is best, in preparing media, to use only non-metal utensils.

In the early days of bacteriology, fluid media were used, but with them it was difficult to obtain pure cultures. The next great advance was made by Koch, who found that many bacteria could grow on a solid medium, and he made the first solid medium by adding gelatin to his broth which caused it, when cold, to set solid. Even when the proportion of gelatin was high this medium became fluid at a temperature much below that of the body, and hence was unsuitable for the cultivation of many of the more delicate bacteria. Agar, obtained from the stems of certain Chinese seaweeds, was substituted for gelatin and a jelly-like medium was then produced which remained solid at temperatures considerably above that of the body. Nutrient agar is the medium now most used in all laboratories, and its introduction is a landmark in the history of bacteriology.

The first step in making medium is the production of a

satisfactory broth or bouillon. There are almost innumerable methods of preparing broth, but only one, that of Wright, will be described as it has been found to be both simple and satisfactory.

### Preparation of Broth

To 1 litre of distilled water add 10 gms. of peptone, 5 gms. of sodium chloride, and 500 gms. of meat, finely minced after removal of excess fat. Horse flesh is cheap and yields a good medium. Mix well and heat for twenty minutes at 68° C., stirring at intervals. Shake well and steam for thirty minutes, filter through paper and adjust the reaction to pH 8.0 (see later). Again steam for thirty minutes and filter through paper. The reaction of the filtrate should now be pH 7.6 : if not, adjust to this. Sterilize, in tubes or flasks, in the autoclave at 10 lbs. pressure for ten minutes.

### Reaction of Media

Pathogenic bacteria are accustomed to live in the body fluids, the reaction of which can change, in the majority of cases, but very slightly from the normal. In order, therefore, to get the best results in artificial culture it is essential that the initial reaction of the medium should approximate to that of blood serum, which is very slightly alkaline.

Hydrogen ion or pH values can be ascertained, for purposes of bacteriology, with sufficient accuracy, by the use of indicators which have different colours at different pH's. For our purpose the most useful indicator is phenol red (phenol sulphone-phthalein), the range of which extends from slight acidity through neutrality to slight alkalinity. The pH of neutrality is 7.0 and of serum about 7.4. A medium having a pH of 7.4 is suitable for the growth of the majority of the pathogenic bacteria. Phenol red is of a yellow colour in acid solutions (pH 6.8) and red in alkaline solutions (pH 8.0) ; at pH 7.4 it is of a pink colour. A range of permanent standards can be purchased which show the colour given by the indicator in the presence of solutions of different pH from 6.8 to 8.0. The colour of the medium interferes somewhat

with the judgment of the colour produced by the indicator ; but this can be obviated by the use of a comparator-box in which the light passes to the eye through the standard tube and a tube of the broth, and can be compared with that coming through a tube of broth containing the indicator and a blank tube with water. Ten c.cs. of broth are measured

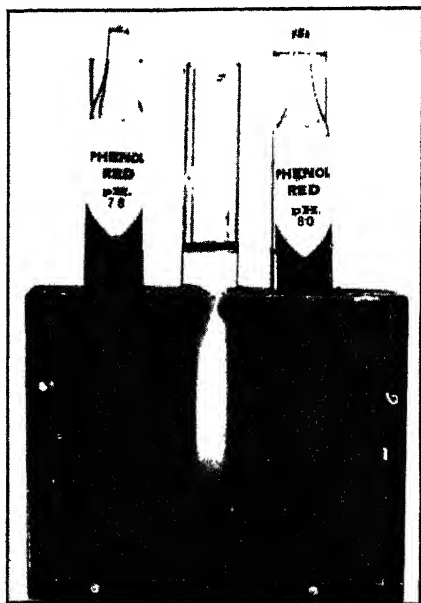


FIG. 10.—COMPARATOR-BOX FOR DETERMINING THE pH OF MEDIA.

into a test tube and 0.5 c.c. of 0.02 per cent. solution of phenol red is added.  $\frac{N}{10}$ -NaOH is dropped in from a 1 c.c. graduated burette until the colour produced in the tube after mixing matches that of the combination of the standard tube (say pH 7.6, if this is the reaction required) and the broth alone. By a simple calculation it is easy to ascertain the amount of  $\frac{N}{1}$  or 10N-NaOH which must be added to the whole bulk of the broth to give it a pH of 7.6.

Organisms, by their metabolism, may produce acids or

alkalis in culture which tend, if permitted to accumulate, to alter the pH of the medium and so inhibit their growth. This is countered to some extent in most media by buffer salts or buffers. These are chemical salts of two kinds: those which, when acted on by acids, form weak acid salts and those which, when acted on by alkalis, form weak alkaline salts. The reaction of a buffered solution responds only slowly, therefore, to the addition of acid or alkali. The buffers most commonly used in media are  $\text{Na}_2\text{HPO}_4$ ,  $\text{NaH}_2\text{PO}_4$  and  $\text{NaHCO}_3$ .

The nutrient broth may be modified in various ways, as by the addition of glucose (0.5 per cent.) or glycerol (5 per cent.). For the growth of many of the more delicate pathogenic bacteria, enrichment of broth by the addition of raw body fluids is essential; 5 to 10 per cent. of sterile hydrocele or ascitic fluid, of blood serum or of citrated blood may be employed.

### **Tubing of Media**

The method of tubing media, whether broth, gelatin or agar in the liquid state or other fluid must be described, since it is of importance to be able to add to each tube the correct amount without the introduction of bacteria and without soiling the upper part of the tube into which the plug fits. To the end of a large funnel is attached, by a short length of rubber tubing, a glass tube drawn out to a moderately fine point. The rubber tubing is fitted with a pinch cock and the funnel is firmly held in a clamp. When it is filled with the medium, the cotton plug is removed from a test tube by holding it between the third and fourth fingers of the right hand and pulling the test tube away from it with a slightly screwing motion of the left hand. The drawn-out glass tube is inserted about halfway down the test tube and the pinch-cock released with the right hand, until the test tube has received sufficient of the fluid, when the flow is stopped. The test tube is cautiously removed to avoid soiling the upper part and the cotton plug firmly replaced.

### Peptone Water

One per cent. of peptone and 0.5 per cent. of NaCl are dissolved in water by boiling. The reaction is adjusted, if necessary, the medium filtered and filled into tubes. This medium is used for testing for the formation of indol by bacteria, and for this purpose the medium should be tried with a known indol-producing organism, since peptone is a rather variable substance. With broth, prepared as described above, indol production is better than when peptone water is used. Peptone water is also used as a basis for carbohydrate media intended for fermentation tests.

### Nutrient Gelatin

To broth add 10 to 15 per cent. of leaf gelatin; dissolve by heating in the steamer for half an hour, adjust the reaction to pH 7.4, cool to about 50° C., and, for each litre, add the white of two eggs beaten up with a little water. Steam for one hour and filter. The coagulated albumin entangles the fine particles in suspension and, with them, is removed by filtration, which is done through filter paper. Since the medium sets at from 22° to 24° C., it must be kept warm during the process. This can be accomplished by keeping the filter and the receiving flask in the steamer or by using a funnel with a hot water or steam jacket. The filtered medium is filled into tubes which may be used for stab-cultures or for pouring plates. Gelatin must be sterilized in the steamer by the intermittent method, as autoclaving robs it of its setting property.

### Nutrient Agar

Agar powder (20 gms. per litre) is added to broth and the whole is heated in the steamer for about two hours. The reaction is adjusted to pH 7.4, and the medium is again steamed for one hour. Filtration, which must be done in the steamer or jacketed funnel, may be through filter paper or, if slight turbidity is not objectionable, through cotton wool arranged in the funnel. When a very clear medium is necessary, it may be cleared with egg as in the case of gelatin.

The filtered agar is filled into tubes either for stabs or for slopes and these are sterilized in the autoclave.

"Stabs" are test tubes containing from 15 to 20 c.cs. of agar or gelatin. They derive their name from their use in making a culture by stabbing the medium from the top towards the bottom with a straight platinum wire inoculated with the material containing the bacteria. They are, however, more generally used for storing agar until it is required for the pouring of plates. The amount given is about correct for a plate 10 cms. in diameter. If a considerable number of plates are frequently required at one time the agar may be stored in flasks or in six ounce, screw top medicine bottles holding about 120 c.cs., sufficient for six plates. "Slopes" or "slants" are generally used for the growth of bacteria already isolated in pure culture. The tubes containing about 5 c.cs. of melted agar are arranged on a flat bench on which rests a bar of wood or glass tube about 2 cms. in thickness. The upper part of the tube rests on the bar, the bottom on the bench itself. The medium becomes solid in this position; but it is advisable to leave the tubes undisturbed for about twenty-four hours, when they can be stored upright. The agar is then found to form a smooth layer reaching from the bottom about one-third of the way up the tube.

Agar may be modified in the same way as broth by the addition of glucose (0.5 per cent.), glycerol (5 per cent.) or other substances.

### Coagulated Serum

This is a useful medium, particularly for the growth of *C. diphtheriæ*. A suitable vessel, such as a large enamelled pail with a lid, is sterilized in the autoclave. It is taken to a slaughter-house where a horse or ox is to be killed and is two-thirds filled with the animal's blood. Absolute sterility is not essential, but every effort should be made to prevent unnecessary contamination. The vessel is left undisturbed till the next day, when it is brought to the laboratory. If the clot is adherent to the sides of the vessel, it is freed with a sterile glass rod and the vessel is again left over night in



a cool place. It will then be found that the clot has contracted and expressed a considerable amount of serum. This is aspirated off and the cells removed, either by centrifuging or by sedimentation in the ice chest. The clear serum may be used for the preparation of medium at once, or may be preserved for future use by the addition of about 10 per cent. of ether. To 4 parts of the serum is added 1 part of glucose broth, and the mixture is filled into sterile tubes, about 5 c.cs. in each for tubes measuring 5 ins. by  $\frac{1}{2}$  in. The tubes are placed in the inspissator, which is a water-jacketed chamber capable of being heated by means of a gas burner. The tubes rest on a rack, which holds them in a sloped position so that the fluid spreads about one-third of the way up their sides. The inspissator is gradually heated to 75° C., at which temperature it is kept for about two hours, the temperature is then brought slowly to about 95° C. when the gas may be extinguished. The serum will then have set into a white or light yellow-coloured solid of firm consistency. It is sterilized in the steamer by the intermittent method. This is usually known as Löffler's medium, although Löffler prepared his medium in a slightly different manner.

### **Tellurite Medium**

Of the many tellurite media for the isolation of *C. diphtheriae*, we have found none more easily prepared or reliable than that of Horgan and Marshall, as slightly modified by O'Mearast. Parish and by others.

Mix 24 c.cs. of a 2 per cent. solution of potassium tellurite, 60 c.cs. of citrated blood (human, ox or sheep) and 15 c.cs. of a sterile 10 per cent. solution of glucose. Add this mixture to 400 c.cs. of agar, melted and cooled to 60° C. Mix and steam for ten minutes, after which the medium is poured into plates or into wide tubes for slopes, in which it is allowed to set.

This is a selective medium which inhibits the growth of the majority of micro-organisms likely to be found in the throat except diphtheria and diphtheroid bacilli, *Oidium* and a few

cocci. Colonies of diphtheria bacilli are black and, when picked off, the subjacent medium is white. This is not so with colonies of most diphtheroids. Since diphtheria bacilli grown on this medium are atypical in morphology, it is always necessary to subculture from it to coagulated serum for further examination.

### Media Containing Indicators

An important point in the identification of many bacteria is their ability to ferment carbohydrates (usually called "sugars," although some of these are really alcohols and others glucosides). The most commonly used carbohydrates are lactose, glucose, maltose, saccharose, dulcitol, mannitol, and inulin. The carbohydrate media may be distinguished by having the plugs of the tubes coloured differently or by inserting in the tube a coloured glass bead, but unfortunately different laboratories have different colour conventions. The basis of the "sugar" media is peptone water. To this is added 1 per cent. of the carbohydrate (except dulcitol, of which 0.5 per cent. is used), and an indicator to show any change of reaction which may occur as the result of bacterial growth. Litmus and neutral red are frequently employed, but we have found Andrade's indicator much superior to these. It is prepared by decolorizing a 0.5 per cent. aqueous solution of acid fuchsin with sodium hydrate solution. One per cent. of the indicator is added to the carbohydrate medium and its reaction adjusted, if necessary, so that the medium is of faint yellow colour. It is then filled into Durham tubes—*i.e.* test tubes on the bottom of which rest inverted very small test tubes. The latter, when the fluid is added, float owing to the air enclosed in them. The

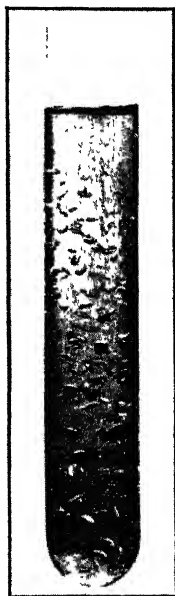


FIG. II.—GAS PRODUCTION IN GLUCOSE AGAR ( $\times \frac{1}{2}$ ).

medium in the tubes is sterilized in the steamer by the intermittent method, never in the autoclave, as high temperatures are very liable to alter the carbohydrates. As a result of the steaming the air is expelled from the inner tubes and the fluid fills them completely. A bacterium grown in one of these media may not have any effect on the carbohydrate, or it may produce an acid reaction alone, or an acid reaction accompanied by the evolution of gas (hydrogen and carbon dioxide chiefly). The production of acid is shown by the medium turning a bright red colour (if Andrade's indicator has been used), and the evolution of gas by the collection of a bubble at the top of the inner tube. (*See Plate IV.*) A solid medium (agar) containing the carbohydrate and an indicator is sometimes employed in examining the fermentative capacity of bacteria (*see Fig. 11*). The tubes are inoculated when the medium is fluid, and this is then allowed to set. After incubation acid production is shown by a change in the colour of the indicator, and evolution of gas by the breaking up of the medium or the collection in it of bubbles of gas. When the amount of gas formed is small, it may be more readily detected in this than in a fluid medium in Durham tubes.

With certain organisms (*e.g.* pneumococci, streptococci, diphtheria bacilli) the broth or peptone water carbohydrate medium does not give very good results, owing to the poor growth obtained. It may, however, be rendered suitable by the addition, to each tube of the sterilized medium, of one-fifth its volume of sterile hydrocele or ascitic fluid or serum. Such tubes should be incubated for twenty-four hours at 37° C. in order to detect any which have become contaminated.

In the testing of water or other fluid for the presence of *Bact. coli*, MacConkey's lactose bile-salt broth is frequently used; 0.5 gm. of sodium taurocholate and 2 gms. of peptone are dissolved in 100 c.cs. of water by boiling and the reaction roughly adjusted. When cold, the solution is filtered and 1 per cent. of lactose and 1 per cent. Andrade's indicator added. The medium is tubed in Durham tubes and sterilized in the steamer.

Milk, freed from cream, and with the addition of an indicator such as litmus solution, is occasionally useful in the identification of bacteria, since certain types produce in it a change of reaction from alkaline to acid, with or without the occurrence of clotting. It should be tubed and sterilized by steaming.

### **Media for the Isolation of Pathogenic Members of the Enteric, Salmonella and Dysentery Groups**

Very many media are used to facilitate the isolation of pathogenic intestinal bacilli from materials, such as fæces, which contain coliform bacilli. These may be divided into those which are differential and those which are selective. The former permit the growth both of coliform bacilli and of the pathogens, but render it easy to distinguish the two types. The latter, more or less completely, suppress the growth of coliform bacilli. We give the formula for only one differential medium, MacConkey's, because we have not found any of the newer differential media to equal it. The formulæ of two selective media are given: with them we have had good results. MacConkey's medium permits the growth of coliform, enteric, salmonella, and dysentery bacilli. The selective media here given suppress the growth both of coliform and of dysentery bacilli. They are, therefore, useful only for the isolation of enteric and salmonella bacilli.

**MacConkey's Medium.**—To 1,000 c.cs. of peptone water add about 5 gms. sodium taurocholate (the exact amount depends on the particular preparation used, some inhibiting the growth of the pathogenic members of the group more than others) and 20 gms. of agar powder, and autoclave for 1 hour. Adjust the reaction to pH 7.4, cool to 50° C., and add the white of two eggs. Autoclave again for 1 hour and filter. Then add 10 gms. of lactose and 2.5 c.cs. of a 1 per cent. aqueous solution of neutral red. This medium is filled into deep tubes for the subsequent pouring of plates and is sterilized in the steamer by the intermittent method. When plates are poured and the medium has set, they should be

left uncovered for about an hour in the incubator, inclined against the sides with bottoms upward in order to give a dry surface to the medium. The object of the sodium taurocholate is to inhibit organisms not accustomed to a habitat in the intestine. *Bact. coli*, as a result of the fermentation of the lactose, produces red colonies; the growth of the pathogenic bacteria of the group does not alter the brown colour of the medium. (*See Plate III.*)

### Wilson and Blair's Medium

The following are prepared :—

- A. 6 gms. Bismuth ammonio-citrate (scale preparation, B.D.H.) are dissolved in 50 c.cs. of boiling distilled water. The solution is neutralized with 10 per cent. NaOH solution (about 2 c.cs. are required), litmus paper being used as indicator; a white precipitate forms. The whole is cooled.
- B. 20 gms. Sodium sulphite crystals are dissolved in 100 c.cs. of boiling distilled water and cooled.
- C. Mix A. and B., add 10 gms. anhydrous  $\text{Na}_2\text{HPO}_4$ , boil for 2 minutes and cool.
- D. 10 gms. glucose are dissolved in 50 c.cs. of water. Boil and cool.
- No. 1. Mix C. and D. This mixture keeps for months.
- No. 2. A solution of 8 per cent. ferrous sulphate in water. This keeps for months and alterations in its appearance are of no importance.
- No. 3. A 1 per cent. solution of Brilliant Green (Grübler) in water. This keeps for months.

To make the medium, to 100 c.cs. of nutrient agar, melted and cooled to about  $80^\circ\text{C}$ ., add 20 c.cs. No. 1, 1 c.c. No. 2, and 0.5 c.c. No. 3, these being shaken before being measured to ensure that the deposits are included. The medium is poured into plates which are dried in an incubator before use.

Almost our only objection to this medium is the difficulty of its preparation. This is eliminated by the use of "Bacto

Bismuth Sulphite Agar Dehydrated," manufactured by the Difco Laboratories. Add 5.2 gms. of this powder to 100 c.cs. of cold distilled water in a beaker. Bring to boiling point over a bunsen flame, with constant stirring, and simmer for two minutes. Cool slightly and pour into plates. The amount stated is sufficient for five plates. The plates should be dried in an incubator before use. By whichever method prepared, the plates should be used on the day they are made.

Two suspensions of faeces, one heavy and the other light, are made in broth (or in tetrathionate broth, described below) and a loopful of each is at once spread over the surface of a plate of the medium. These are incubated, preferably for forty-eight hours.

*Bact. coli* is completely suppressed as also are most coliform bacilli except *Bact. aerogenes*, which produces brown sticky colonies. The dysentery bacilli are also suppressed. *Bact. typhosum*, the paratyphoid bacilli and most other salmonella bacilli grow freely on the medium. When the colonies are well separated from one another, they are flat, black, and dry, and the surface of the medium surrounding them develops a metallic sheen. If the colonies are very close together, they are small and green in colour.

**Tetrathionate Broth.**—To 90 c.cs. of broth add 2.5 gms. of chalk, previously autoclaved and dried. Steam for half an hour. When cold, add 10 c.cs. of a 60 per cent. solution of crystallized sodium thiosulphate which had been steamed for half an hour and cooled and 2 c.cs. of a solution prepared by dissolving 6 gms. of iodine and 5 gms. of potassium iodide in sufficient distilled water to make 20 c.cs. The mixture is well shaken and distributed into sterile tubes, 5 c.cs. per tube.

A tube of the medium is heavily inoculated (0.5 gm.) with faeces and a second lightly (one loopful). Both are incubated over night. A loopful from each is plated on MacConkey's medium and the plates incubated. It is customary to find, on one or both plates, after incubation, a pure or almost pure growth of typhoid, paratyphoid or other salmonella bacilli, if the faeces contained one of these organisms.

### Media for distinguishing Coliform Bacilli

**Buffered Glucose Broth.**—Add 5 gms. peptone (Proteose-peptone, Difco or Witte's), 5 gms. glucose and 5 gms.  $K_2HPO_4$  to 800 c.cs. of water. Dissolve by heating in the steamer, filter, cool, and make the volume up to 1,000 c.cs. This is filled into tubes, 10 c.cs. to each, and sterilized by steaming on three successive days. This medium is used for the methyl-red and Voges-Proskauer tests.

**Koser's Citrate Medium.**—Dissolve 1.5 gm. sodium ammonium phosphate, 1.0 gm.  $KH_2PO_4$ , 0.2 gm. magnesium sulphate, and 2.5 gms. sodium citrate (crystals) in 1 litre of water. This is filled into tubes (about 5 c.cs. to each) and autoclaved. This medium is used to determine whether coliform bacilli can grow in it (as does *Bact. aerogenes*) or not (*Bact. coli*) when the bacteria are lightly inoculated into it. Growth is judged by the development of turbidity.

### Griffith's Egg Medium

Fresh eggs are scrubbed, immersed in boiling water for ten seconds and then in spirit. When dry, they are opened, with aseptic precautions, and the contents transferred to a sterile graduated cylinder. To the eggs is added one-half their volume of sterile saline. The mixture is shaken, with a rotary movement, in a sterile flask for twenty minutes and is filtered through sterile gauze into a sterile flask. The egg-saline mixture is filled into sterile tubes, 5 c.cs. in each. The medium is inspissated in a sloping position at 80–85° C. for forty-five minutes. Next day it is held for 1½ hours at the same temperature. The plugs should be treated with melted paraffin wax to prevent drying of the medium. Tubes should be tested for sterility by incubating at 37° C. for three days.

Glycerol egg medium is prepared as above, except that sufficient glycerol is added to the egg-saline mixture to give a concentration of 5 per cent.

### Robertson's Medium for Anaerobic Bacteria

To 500 gms. of fresh, minced beef add 500 c.cs. of boiling  $\frac{N}{20}$ -NaOH and simmer for 20 minutes. Strain through cloth

and discard the fluid. Spread the meat on filter paper to dry. When dry put the meat in tubes to the depth of about 2.5 cms. and cover with sufficient broth (pH 7.6) to form a layer about 5 cms. deep. Heat in water bath at 100° C. for half an hour to expel air, cover with a layer of liquid paraffin 1.0 cm. deep, plug with wool and sterilize in the autoclave for one hour at 115° C. The tubes should be heated in boiling water for a few minutes to expel all dissolved oxygen and then cooled before using if they have been stored for some time after preparation.

### **Hydrocele or Ascitic Fluid**

For the enrichment of media, sterile hydrocele or ascitic fluid is very useful. If the instruments and vessels are sterilized before the operation, the fluid may be transferred by means of a sterile pipette into test tubes (20 c.c.s. in each), in which it is stored until required. These should be incubated or forty-eight hours in order to detect any contamination. If the fluid has not been collected aseptically, it may be freed from bacteria by filtering through a Chamberland or Seitz filter, after which it is stored as described above.

### **Blood**

Agar or broth, enriched by the addition of serum or whole blood, are among the most commonly used media. Blood may be obtained from the rabbit by heart puncture. The blood is drawn into a sterile 10 c.c. or 20 c.c. syringe from the anæsthetized animal, the needle having been inserted through the left anterior chest wall, previously shaved and treated with tincture of iodine, in the interspace next above that at which the apex beat is best felt. If the operation is carefully performed the animal survives without apparent injury. In laboratories where the amount of such medium used is not excessive, or where patients attend to be bled for the Wassermann test, human blood will be found simpler to obtain. An all-glass syringe (20 c.c.), prepared as described on page 107, may be used. The region of the bend of the elbow is treated with tincture of iodine and a tourniquet moderately tightly



applied above it. If no large vein is seen, allowing the arm to hang down and clenching the fist may render some prominent. The skin is pulled tightly to one side, the needle inserted through the skin to one side of the selected vein and then, in a second motion, into the vein. The piston is cautiously withdrawn, filling the syringe with blood. The tourniquet is removed and the needle withdrawn. If the operation has been correctly performed, no bleeding will occur and no dressing is necessary. The syringe is emptied into a test tube containing 4 c.cs. of sterile 3·8 per cent. sodium citrate to prevent clotting, and the contents are mixed by rotating the tube between the hands. This citrated blood will keep in good condition in a cool place for about a week. The cells sink to the bottom and should be well shaken up before use. If serum is required, the blood should be collected in a dry sterile test tube and allowed to clot. The clot may be loosened from the sides of the tube with a sterile wire, and when it contracts the serum will be squeezed out. The cells fall to the bottom and the clear serum may be pipetted off when required. Where only a few blood agar slopes are required, sufficient blood may be collected in a Pasteur pipette from a needle puncture in a finger, previously disinfected with tincture of iodine and then treated with alcohol, the latter being allowed to evaporate before the puncture is made.

### **Sterile Solutions**

In addition to the supply of media it will be found very useful to have a supply of various solutions ready sterilized in plugged tubes or in screw-top medicine bottles. These should include distilled water, physiological saline (0·85 per cent. NaCl), carbol-saline (0·5 per cent. phenol in saline), citrate (3·8 per cent. sodium citrate), and glucose (10 per cent.). It is convenient to render these easily distinguishable without labelling by using wools of different colours as plugs, or by the presence in each of a glass bead of the appropriate colour.

## CHAPTER V

### THE MAKING OF CULTURES

THE microscopic examination of bacteria enables them to be grouped into certain large classes on morphological grounds; but by this method scarcely any organism can be definitely identified. One of the most essential steps in bacteriology is to secure a pure culture of an organism. A pure culture is one in which only a single type of organism is present; it is usually the offspring of a single organism. Before describing the methods used for attaining this end, it is necessary to give some account of the apparatus used in the cultivation and examination of bacteria.

Certain pieces of apparatus, similar to those employed in chemical, zoological, and botanical laboratories, are essential for the study of bacteria. Of these the most important are test tubes, Petri dishes, commonly called plates (which name is a relic of the days when the pioneers in bacteriology made cultures on gelatin medium poured on flat glass plates), flasks, pipettes, graduated cylinders and thermometers. A centrifuge is a necessity in a bacteriological laboratory. Of such machines a large number of types are available, which may be driven by hand, water, or electric power. A hand centrifuge is useful in obtaining the deposit from a urine, for example; but one which can be run at several thousand revolutions a minute is desirable. One of the most efficient and reasonably priced of these is the Lundgren Angle Centrifuge. Every centrifuge should have a free-wheel device to prevent the tubes being brought to rest too rapidly when power is cut off, as this disturbs any deposit formed. It is essential to balance the centrifuge by placing in the opposite bucket a tube similar to the one used and containing the same quantity of fluid.

Very useful in a laboratory is some form of suction

apparatus, of which a good type is a Geissler's vacuum pump. This can be used in filtering and also for withdrawing the supernatant fluid after centrifuging so as to leave the deposit undisturbed.

Probably the most used piece of apparatus is the platinum wire. Several pieces of this wire (or a fairly good substitute, resistance wire) of different thickness and about 6 cms. long are required. These are mounted either in aluminium holders, or by fusing them into glass rods. Some should be left straight and others have their free ends bent into loops. This can be done by moulding the end round a glass rod which has been drawn out in the flame. The loop should be about 5 mm. in diameter, and must be closed so that it can be used for transferring drops of fluid.

Another necessity is a supply of glass tubing about 30 cms. long and 0.7 cm. in external diameter. Both ends are rounded

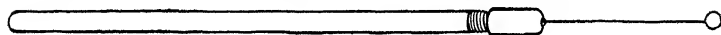


FIG. 12.—PLATINUM WIRE IN ALUMINIUM HOLDER ( $\times \frac{1}{2}$ ).

in the flame, plugged with cotton wool, and the tubes are sterilized in the oven. When a pipette is required for the transference in a sterile manner of a larger quantity of fluid than that held by a platinum loop, one of the prepared tubes is held, one end in each hand, and the central part heated in a Bunsen flame, the tube being rotated all the time. When thoroughly soft, the glass walls fall in slightly and thicken, and then the tube is removed from the flame and the ends drawn slowly apart, the tube being rotated during this operation. When of sufficient length, the centre is again heated and the ends sharply pulled away from one another. This will produce a very fine capillary tube, which is easily broken, and the ends can be sealed in the flame, so protecting the two pipettes thus produced until they are required. A rubber teat is fitted to the plugged end (without removing the cotton) and the capillary portion broken at a convenient length. A well-made Pasteur or capillary pipette of this type should have a length of about 12 cms. unnarrowed, a rapidly

tapering part of about 1 cm. and a very slowly tapering portion of about 10 cms., of which the proximal part will have an external diameter of about 2 mms. and the distal of about 1 mm. If the drawn-out part tapers too sharply, either the tube has been insufficiently heated or the ends have been pulled apart too rapidly. A considerable number of tubes should be prepared at one time and a pair of pipettes made from one of them when required.

Spreaders, the use of which will shortly be described, can be made from thin glass rod, but those made from an iron wire are more permanent and, if kept polished, are quite satisfactory.

When it is desired to transfer bacteria from one culture tube to another, that is, to make a sub-culture, the following is the procedure. The plugs of all the tubes used should first

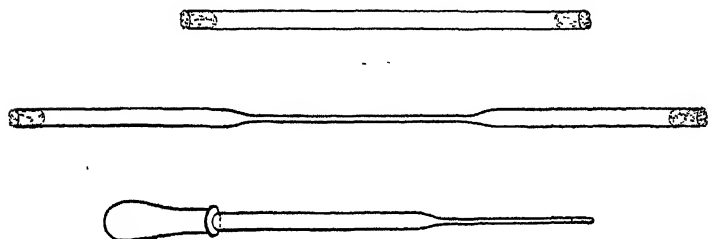


FIG. 13.—THE PREPARATION OF A PASTEUR PIPETTE.

be passed through the flame and the burning cotton extinguished by rapidly pressing it in the hand. This destroys any bacteria which are on the cotton and which otherwise might contaminate the culture. The plugs are then twisted in the tubes to break down any adhesions. The two tubes are held in the left hand, the exact method of holding them being unimportant so long as the medium is not obscured by the hand. Some hold one tube between the thumb and forefinger, the other between the fore and middle fingers, in each case the upper part, about 5 cms. from the top, being grasped. Personally, we prefer to hold the extreme bottoms of the tubes between the tip of the thumb and the bunched tips of the fingers. In this way three or four tubes may be held at the same time radiating like the ribs of a fan. The

projecting portions of the plugs are grasped between the fingers of the right hand near their webs, the interior part of the plugs being directed away from the palm. If necessary two, or even three, plugs may be held between two fingers. After removing the plugs, the mouths of the tubes are quickly passed through the flame to burn off any adherent cotton. The holder of the platinum loop is held like a pen in the right hand and the wire heated to redness, when the lower part of the handle is passed a few times through the flame. The sterilized wire is then introduced into the tube containing the culture. The wire may be cooled, either in the water of condensation in the tube or by touching a part of the medium on which there is no growth. The culture is then touched



FIG. 14.—SPREADER.

with the loop, to which some will adhere. The charged loop is withdrawn and introduced into the other tube, care being taken to prevent it touching the mouths or sides of the tubes. The medium is inoculated, as is described later, the wire is sterilized in the flame, and the plugs replaced, each to its own tube. Throughout the whole operation the tubes should be held as nearly in a horizontal position as the medium permits to prevent the entry of bacteria falling from the operator or those floating in the air.

In all operations in which the use of a platinum wire is involved it is essential to sterilize it twice, once before the material containing the bacteria is touched to prevent the culture being contaminated, and the second time after the culture has been made and before the wire is laid down, to prevent accidental infections. When the loop contains a mass of bacteria or a drop of infected fluid it should first be held some distance above the flame and then gradually lowered, as sudden heating frequently causes spurting, which may lead to contamination of the bench.

Sloped or slanted media are inoculated by drawing the charged loop gently over the surface from below upwards in a straight line or, if the maximum amount of culture is desired, by rubbing the whole of the surface over with the loop. Stabs of agar or gelatin are inoculated with the straight wire which, after it is charged, is pushed gently straight down into the medium in the middle line. In the case of gelatin, if it has been prepared a considerable time before this, a crack is likely to be caused which may interfere with the typical growth characteristics. The remedy is to melt the medium and allow it to set again a short time before it is inoculated. Fluid media are inoculated either by gently shaking the loop in the medium or by emulsifying the material in the fluid on the side of the tube just above the level of the main bulk of the medium and then washing this off by slanting the tube.

Since bacteria rarely occur in a pure state in nature, it is essential to learn the methods of obtaining pure cultures from material containing two or more varieties of bacteria. With the use of fluid media this can be done only in exceptional cases, and both luck and perseverance are necessary.

Solid media allow us to isolate an organism, even when it is enormously outnumbered by bacteria of another variety. The medium is usually used in plates, and cultures may be made either in the medium (deep plating) or on its surface (spread or streak cultures). The principle is that in or on a solid the bacteria are unable to move about. If the food material is satisfactory each organism multiplies and, in the course of some time, the offspring of each are so numerous that the mass becomes visible to the naked eye as a colony. If the individual organisms were sufficiently far apart in or on the medium it is possible to touch a single colony with a sterile wire, which is then used to inoculate a tube of medium, and this, after incubation, will contain a pure culture. The number of plates used when cultures in the depth of the medium are made depends on the number of bacteria in the material: if this is unknown, it is desirable to make several plates. Three tubes of agar are placed in a vessel containing

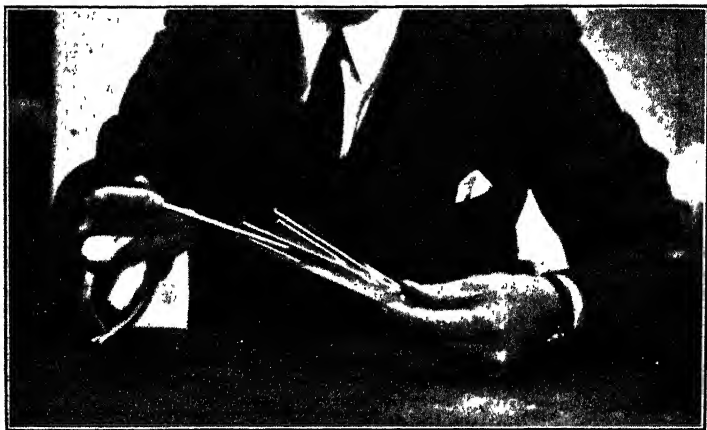


FIG. 15.—THE METHOD USED TO INOCULATE A TUBE OF MEDIUM.  
(Note how the tubes, platinum wire and plugs are held.)

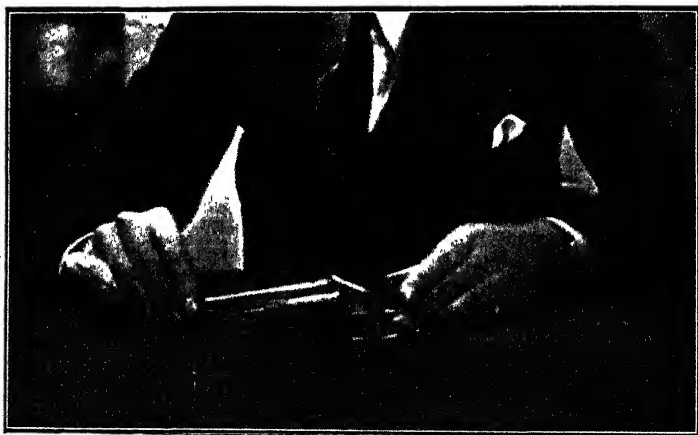


FIG. 16.—“POURING” A PLATE.  
(Note how the lid is held over the plate.)

water, which is boiled for a few minutes until the agar is melted. The water is cooled by the addition of cold water until, after mixing, its temperature is about  $48^{\circ}\text{C}$ . A loopful of the material is transferred to one of the tubes with the usual precautions, and the whole thoroughly mixed by rotating the tube gently between the hands without allowing air-bubbles to form. Five loopfuls of the contents of this tube are transferred to the second, and after the same treatment a similar amount from the second to the third tube. The contents of each tube are poured into sterile Petri dishes (plates) and, after solidification, the plates are incubated. If the material contains a very large number of bacteria the first plate will probably show far too many colonies, but either the second or third ought to give a suitable number for picking. Certain precautions must be taken in pouring the liquid medium, whether containing bacteria or not, into the plates, in order to prevent accidental contamination. The plates should not be removed from the paper in which they were sterilized until the medium is ready. They should then be unwrapped and placed on a horizontal table in a place free from dust. The outside of the tube is dried, the plug is removed, the mouth flamed, and the lid of the plate is lifted at one side just sufficiently to allow the tube to be introduced beneath it and the medium is poured into the plate. The lid is then replaced, and the plate is not moved until the medium is thoroughly set. At no time, either before or after the medium is poured into the plate, is its interior left uncovered by the lid, as this may permit the entry of bacteria from the air. The procedure of plating as described above requires some care and judgment, as it is necessary to avoid over-heating the bacteria, which might kill them, and also to guard against the medium solidifying before the plate is poured. Agar melts slightly below  $100^{\circ}\text{C}$ . and sets at about  $42^{\circ}$  to  $44^{\circ}\text{C}$ . Since the medium was at  $48^{\circ}\text{C}$ . before the infected material was added the work must be done rapidly and the plate poured before its temperature has fallen to  $44^{\circ}\text{C}$ . Where gelatin is used the technique is simpler. The medium may be inoculated at a temperature of about  $35^{\circ}\text{C}$ ., and a considerable time



elapses before it sets at air temperature. Gelatin cultures are incubated at 20° to 22° C.

Agar plates are always incubated in the inverted position, *i.e.* lid downwards. If reversed, the water of condensation would collect on the lower surface of the lid and fall on the medium. Some of the bacteria on or in the medium might grow in this water and spread over the surface, rendering the culture useless. Gelatin plates are, however, incubated with the lid uppermost as, if inverted, any liquefaction of the medium by the growth of liquefying organisms would destroy the culture.

For spread or streak plates the method differs somewhat from the above. The medium is melted and cooled as before and poured into plates. It is always advisable to cool the medium before pouring it, for thus the plate is less likely to be cracked, and also the amount of condensation water on the lid will be less. Plates should not be used for spreading until at least an hour or two after pouring, as then the surface will be both firmer and drier. With a wet plate there is always the risk of an organism multiplying in the fluid and spreading over the entire surface. In the case of plates spread with faeces this is particularly important, since *Bact. coli* grows actively and is motile and so may prevent the isolation of other bacteria. For this reason, plates made of the special media used for isolating pathogenic intestinal bacteria (as MacConkey's medium) must be dried in the incubator before use.

For the routine plating of pus and other morbid fluids from the body blood agar is preferable to plain agar. A tube of agar is melted and cooled to 55° C. About 1.5 c.c. of citrated blood, collected as described in Chapter IV, is added by means of a sterile Pasteur pipette and thoroughly incorporated with the agar, either by rolling the tube between the hands or by pouring into another sterile tube and back again. It is then poured into the plate and allowed to set. A properly made blood agar plate should be uniformly translucent and of a moderately deep red colour. A simpler alternative is to place the necessary amount of blood in the plate and pour on

it the melted and cooled agar. The covered plate is then lifted in both hands and rocked so as to diffuse the blood throughout the medium. This operation must be completed before setting commences, as otherwise an uneven surface will result. This method never gives such a uniform mixture as that described above, but the risk of accidental contamination is less and the plates are quite satisfactory for routine use. Plates of agar enriched with serum, hydrocele or ascitic fluid are made in exactly the same way. Glucose-agar may be prepared by adding one-tenth volume of a sterile 10 per cent. solution of glucose to the melted agar before pouring. Where agar, enriched with blood, is required and no adequate supply of blood is available, a few drops of blood, taken with aseptic precautions from a finger and spread on the surface of an agar plate or agar slope, will permit the growth of some of the more delicate organisms which would not grow on plain agar. This medium is described as "blood-smeared agar."

Plates intended for spread or streak cultures should always be used on the day on which they are made. In pouring the plate there is the possibility of a bacterium falling on the surface of the medium. If the plate is used fresh, a single contaminating colony will result, and this can generally be distinguished without difficulty from the colonies due to the bacteria in the material under investigation. If, however, the plate has been kept from the previous day, the bacterium may have produced a colony too minute to be seen with the naked eye or hand lens, but nevertheless consisting of thousands of bacteria. In the spreading operation these bacteria are distributed over the surface of the plate, where they give rise, on subsequent incubation, to a very large number of colonies which obscure those of the causative bacteria.

Two methods are employed for inoculating the prepared plates. In the first—the method of parallel streaks—the platinum loop is charged with the material and drawn lightly over the surface of the medium in a series of parallel lines about 1 cm. apart. The loop is not re-charged between the drawing

of each line and, during the operation, accidental contamination is avoided by holding the lid above the plate in the left hand. After incubation, the first streak made will probably show a uniform line of growth in which isolated colonies are not seen: in the later streaks the growth is less heavy and, usually in the third or fourth, isolated and well-separated colonies are produced.

A spread method is better than that of streaks for the



FIG. 17.—THE COLONIES ON A PLATE SPREAD BY THE METHOD OF PARALLEL STREAKS ( $\times \frac{2}{3}$ ).

isolation of colonies. A loopful of the material is placed near the edge of the plate and the spreader sterilized in the flame and allowed to cool. Before it is used it should touch the water of condensation on the lid to make quite certain that it is not too hot. With the spreader the drop is well rubbed over a small portion of the surface, then the spreader is lifted and rubbed over a fresh area. This is repeated until the whole of the surface has been rubbed. A good plate—that is one showing on some part well-isolated colonies—is usually secured if about seven or eight areas are rubbed in turn, the spreader never being returned to an area previously touched.

Occasionally better results are obtained by increasing the area devoted to each rubbing and using two plates instead of one. During spreading the plate may be protected from accidental contamination in the same way as was advised in the description of the method of parallel streaks, the spreader being manipulated under the raised lid, or the lid may be removed and the plate held in the left hand, the spreader being in the right. If the plate is held with its surface vertical, any falling



FIG. 18.—THE COLONIES ON A PLATE INOCULATED BY SPREADING.

bacteria will rest on the upper exterior surface of the rim and the medium will escape.

The period of incubation after which the plates are in best condition for obtaining pure cultures depends on the variety of organism cultivated and also on the method and material used. Usually, 24 to 72 hours' incubation will give a satisfactory plate. The process of obtaining pure cultures from a plate containing colonies of several varieties of bacteria is known as picking or fishing. The plate must first be carefully examined with the naked eye and a hand lens, both from above and by holding it up to the light, and the apparently different varieties of

colonies noted. The lid is removed and the plate held in the left hand with its surface vertical. In the right hand is held the platinum wire, straight for minute colonies or with a loop for those of larger size. The wire is sterilized, cooled by touching a part of the medium devoid of growth, and the colony touched with it. The plate is covered and the wire rubbed over the surface of an agar slope or used for inoculating a tube of some other medium. This tube, after incubation, should contain a pure culture of the bacteria present in the colony picked. The operation may be facilitated by the use of a watchmaker's lens held in the eye. The picking of the deep colonies obtained when the material was mixed with the melted medium is similar, except that in this case the wire must penetrate the medium to reach the colony required. In doing so it is necessary to avoid any colony lying above or below the one aimed at.

In the majority of cases the use of one or other of these plating methods will secure pure cultures, but occasionally certain varieties of bacteria, particularly the anaerobic bacilli, appear to cling closely together, and so a single colony may arise from two individuals which may have been of different varieties. This difficulty may often be overcome by repeated plating, a single colony being plated out on a fresh plate, one colony from this plated on another plate, and so on.

In artificial culture two phenomena may occasionally be noticed which are probably of greater importance to bacteria growing in their natural habitat than to those in culture. The first of these is symbiosis, that is the living together of two or more varieties of bacteria, the growth of one assisting the development of the other. As has just been mentioned, this very commonly occurs with the anaerobes. Other examples are a mixed growth of an obligatory aerobe (such as *B. subtilis*) with an anaerobe, the former utilizing the oxygen which would be harmful to the latter, and the increased size of colonies of *H. influenzae* in proximity to a colony of a staphylococcus. In the opposite phenomenon, antagonism, the growth of one species inhibits the growth of the other. If the medium contains a carbohydrate fermentable by one,

the acidity developed may inhibit the growth of the second : *L. acidophilus* in milk culture ultimately destroys the putrefying bacteria, *Ps. pyocyanea* inhibits the growth of the gonococcus and streptococci prevent the development of *Past. pestis*.

Where one particular type of organism is believed to be present in a mixture, a knowledge of the peculiarities of its structure or culture may suggest a method for its easy isolation, even when it is present in such small numbers as to make its separation by ordinary plating rather unlikely. As instances of this we may mention the use of the medium of Horgan and Marshall in growing *C. diphtheriæ* and the employment of Wilson and Blair's medium in detecting the presence of enteric bacilli in fæces. The cholera vibrio grows well in a very alkaline medium and is strictly aerobic, and so we add fæces, suspected to contain the vibrio, to alkaline peptone water and, after incubation, we find on the surface an almost pure culture of this organism. Streptococci grow abundantly and quickly in glucose broth, and form a deposit in the bottom of the tube which, when plated, will usually permit of their isolation, even when the original material contained a very small number. Many more examples of the use of such, more or less selective, media to assist in the obtaining of pure cultures might be given. The isolation of a sporing bacillus from non-sporing bacteria is simple since, by selective heating, the latter can be killed leaving the former still viable.

It is customary, when a colony is picked off a plate, to inoculate an agar or blood-agar slope with it, and so obtain a pure culture. When this culture has grown, sub-cultures may be made from it in media suitable for the identification of the organism, such as those containing various carbohydrates.

When cultures of very slowly growing organisms are made on sloped media in tubes, precautions must be taken to prevent drying, which would interfere with growth. The plug may be cut off level with the top of the tube, which is covered with an india-rubber cap, or the plug may be immersed in melted paraffin wax. Neither of these methods is entirely satisfactory

since they interfere with the free supply of oxygen. The tubes may be placed in a cylindrical vessel with a lid, some water being present in the bottom of the vessel and the whole incubated. The growth of moulds, which may penetrate the plugs, can be prevented by moistening the projecting part with  $\frac{1}{5000}$  mercuric chloride solution.

All cultures should be fully labelled with the source, name of organism, and date on which the culture was made, as soon as the medium has been inoculated. Stick-on labels are unsatisfactory and dangerous; grease pencils are better.

The methods already described are applicable chiefly to the aerobic bacteria. For the cultivation of anaerobic bacteria, which grow only in the absence of oxygen, special procedures must be adopted. Either ordinary culture media, liquid or solid, in plates or tubes, may be used, these being contained in a special apparatus, or else media adapted for the growth of these organisms without any new apparatus. The principles of the various methods are: (1) exclusion of air; (2) exhaustion of air; (3) absorption of oxygen from the air; or (4) replacement of air by a neutral gas, such as hydrogen or nitrogen, but not by carbon dioxide, which inhibits the growth of many species of bacteria.

The majority of anaerobic organisms are capable of growing in the deep parts of solid media either agar or gelatin, especially if these contain a reducing substance such as glucose. The tube of medium is immersed in boiling water for five or ten minutes which liquefies it and expels any dissolved oxygen. It is cooled rapidly to 50° C. for agar, or 30° C. for gelatin, and inoculated, the added bacteria being diffused by rolling the tube without shaking, which would allow oxygen to be absorbed. It is then cooled and incubated in the ordinary way. Anaerobic bacteria produce colonies in the lower part of the tube and those with minimal oxygen requirements show densest growth in a layer 0.5 to 2.5 cms. from the top of the medium. Anaerobic organisms may be separated from one another by this method, the colonies being picked after the removal of the cylinder of medium from the tube. This is done by cutting around the tube with a file or diamond

at about the level of the middle of the column of medium. The exterior of the tube is sterilized with antiseptic solution, which is washed off with alcohol, the latter being burned off. The tube is broken by applying a red hot wire or glass rod to the mark, when a crack will develop along the mark. The lower portion of the tube is removed and the cylinder of medium caught in a sterile Petri dish, where it can be examined and colonies picked.

Anaerobic bacteria frequently grow at the bottom of a deep tube of glucose broth, which has been heated in boiling water to expel air and cooled before use. Fluid cultures are more successful, however, if the surface is covered with a layer of liquid paraffin and the medium heated to 100° C. for a few minutes and rapidly cooled before it is inoculated with the bacteria, which must be done with a capillary pipette. The paraffin is theoretically an imperfect seal against oxygen, but in practice it works well. Melted vaseline is probably superior to paraffin for this purpose. The presence of portions of fresh sterile animal tissue (rabbit kidney) in a fluid medium greatly promotes the growth of anaerobes, and this is utilized in Noguchi's method for the cultivation of spirochætes. Even cooked meat assists and, for the cultivation of anaerobic bacteria, Robertson's medium—which contains fragments of minced meat, the whole being covered with paraffin—is very satisfactory. The method of removing some of the culture for microscopic examination from a fluid medium covered with oil must be described. A platinum loop is useless, as satisfactory preparations cannot be made from a mixture of medium and oil. With the usual precautions against contamination, some of the medium is aspirated into a capillary pipette, care being taken not to draw up any of the oil. The terminal portion of the pipette is then heated in the Bunsen flame and the oil on its exterior burned off. When the glass is cool, a few drops of the medium are expelled into disinfectant solution in order to wash out the portion of the pipette heated, and then drops are expressed on to slides for the preparation of films.

The many varieties of apparatus formerly employed for



anaerobic culture, which relied chiefly on the replacement of air by hydrogen and the absorption of oxygen by alkaline pyro solution, have now been almost entirely superseded by the McIntosh and Fildes electrically heated anaerobic jar, which robs anaerobic methods of much of their difficulties. It consists of a glass or metal jar and a heavy metal lid which is fixed to the top of the jar by a clamp, an air-tight join being effected with vaseline, plasticine, or a rubber washer. The lid is pierced

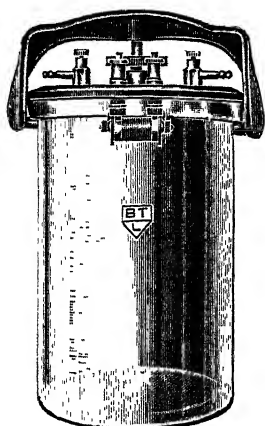


FIG. 19.—THE MCINTOSH AND FILDES ELECTRIC ANAEROBIC JAR.

(Messrs. Baird & Tatlock  
(London), Ltd.)

by two screw-down valves and two electric terminals. In the interior, suspended from the terminals, is a little roll of copper gauze containing asbestos wool holding finely divided palladium. A resistance wire, connected with the terminals, surrounds this so that, when an electric current passes, the palladium is heated. The cultures are placed in the jar and the lid carefully adjusted and clamped. One valve is connected to a hydrogen supply (either a cylinder, in which case the gas must be passed through an apparatus for reducing pressure, or an apparatus in which hydrogen is generated from zinc and sulphuric acid and purified by passing through wash bottles containing solutions of lead acetate, silver nitrate and alkaline pyro to absorb  $H_2S$ ,  $AsH_3$  and oxygen). With both valves open, hydrogen is sent through the jar for some time until, when the gas escaping from the second valve is collected in an inverted test tube and ignited, it burns without exploding. The escape valve is then screwed down, the other being left open to admit hydrogen. The electrical terminals are connected to the supply through a resistance composed of carbon lamps, the number and power of which depend on the voltage. The

current heats the palladium which, when hot, acts as a catalyst assisting the hydrogen to combine with the last trace of oxygen in the jar. The current is passed for about twenty minutes (it requires a considerable time to eliminate all oxygen contained within test tubes and plates) and is then switched off, the valve leading to the hydrogen supply being left open until the jar has cooled when it is closed, the hydrogen supply disconnected and the jar incubated. An indicator may be used to show that anaerobic conditions are maintained. It is composed of equal parts of—(a) N/10 NaOH 6 c.cs., water to 100 c.cs.; (b) 0.5 per cent. watery methylene blue 3 c.cs., water to 100 c.cs.; (c) glucose 6 gms., water to 100 c.cs. and a crystal of thymol. The mixture, in a test tube, is boiled thoroughly until colourless and put into the jar with the cultures. It remains colourless so long as oxygen is excluded, but turns blue in the presence of very slight traces of that gas.

In dealing with anaerobes, the greatest difficulty is experienced in obtaining pure cultures. Ordinary plating methods, which usually succeed with aerobic organisms, frequently fail with the anaerobes, chiefly owing to the marked preference which these have for symbiosis: two individuals apparently cling together and produce a mixed colony. In sub-cultures the two grow at a fairly uniform rate, neither outgrowing the other. It has frequently been found that a believed pure stock culture of an anaerobic bacterium, which may have been cultivated for years in a laboratory, really consists of two distinct species. This fact explains the great divergence which formerly existed, and even now exists although to a less extent, in the standard descriptions of the characteristics of certain anaerobes. Repeated plating, differential heating, by which less resistant bacteria or spores are destroyed, dilution methods and animal inoculation, if practised with great care and skill, may succeed in the production of a pure culture; but the only reliable method is by the isolation and culture of a single bacterium or spore actually seen with the microscope at the moment of its capture. The methods of doing this (such as that of Barber) are difficult and extremely tedious,

and are impossible for the student, or indeed for any one who has not devoted a considerable time to mastering the details of the technique.

Certain bacteria, especially *Brucella abortus* and the gonococcus, grow best in an atmosphere containing about 10 per cent. of carbon dioxide and others, such as staphylococci, produce maximum amounts of toxin in the presence of about 20 per cent. of this gas. For test-tube or plate cultures of such bacteria, the necessary conditions are most easily provided in a jar, such as that of McIntosh and Fildes. The mixture of carbon dioxide and air

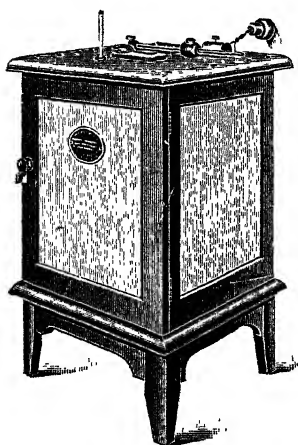


FIG. 20.—INCUBATOR.  
(Messrs. Hearson.)

or oxygen may be forced into the jar under pressure through one of the valves, so displacing the jar's original content of air through the other. Alternatively, carbon dioxide may be produced in the jar by adding acid to a tube containing a weighed amount of sodium bicarbonate immediately before the lid is closed (1 gm.  $\text{NaHCO}_3$  produces 260 c.cs. of  $\text{CO}_2$ ) or by placing in the jar a weighed amount of carbon dioxide snow (1 gm. yields 500 c.cs. of the gas).

For the cultivation of bacteria some apparatus for maintaining the cultures at a constant temperature is almost essential. The optimum temperature of growth for the majority of human bacteria is that of the body,  $37^\circ \text{C}$ . For gelatin medium a temperature of about  $20^\circ$  to  $22^\circ \text{C}$ . is suitable. The apparatus is known as an incubator, and those manufactured by Messrs. Hearson are very simple and reliable. They consist of a square metal box with, on five sides, double walls, and on the sixth a glass door. The whole is contained in a wooden case between the walls of which and those of the metal box is

placed a layer of insulating material. A door corresponds with the interior glass door and the bottom of the metal box is not encased. The hollow metal walls contain water which is heated, either by gas, electricity, or an oil lamp. The most important point is the regulating device which automatically turns down the supply of gas, switches off the current or diverts the heat of the lamp, when the interior has reached the desired temperature. When the temperature falls, the heat is again applied. The mechanism is controlled by the "capsule" which is situated within the incubator. This is a thin metal box, measuring 5 cms. square by about 0.5 cm., in which is hermetically sealed some volatile liquid, the nature of which depends on the temperature required. When the capsule is heated to a temperature above that of the boiling point of the fluid, the vapour pressure greatly increases and the sides of the capsule bulge out. This increase in thickness is utilized by a system of levers to lessen or cut off the supply of heat. On cooling, contraction occurs and heat is again applied. A movable weight on a rod enables varying pressure to be applied to the capsule, and thus gives a fine adjustment by which the temperature can be exactly regulated. Once the incubator has been regulated, it works satisfactorily for years, and the only attention required is the occasional addition of water to make up for that lost by evaporation. Plates can be stacked in the incubator, with the medium upwards (except in the case of gelatin), and tubes can be held either in racks, in wire baskets or, a convenient substitute, tobacco or cigarette tins. The door of an incubator should never be left open, and indeed should only be opened for the time necessary to place in or remove from the incubator the cultures required. A mechanism somewhat similar to that used in incubators may also be applied to water-baths which are frequently used in bacteriological laboratories. At least four of these, adjusted to work at 37°, 52°, 55°, and 60° C. are essential.

## CHAPTER VI

### SPECIAL TECHNIQUE

IN this chapter it is proposed to describe the technique of certain operations carried out in the laboratory.

#### Filtration

Filtration in the bacteriological sense is employed to obtain water or other fluids free from bacteria for domestic or

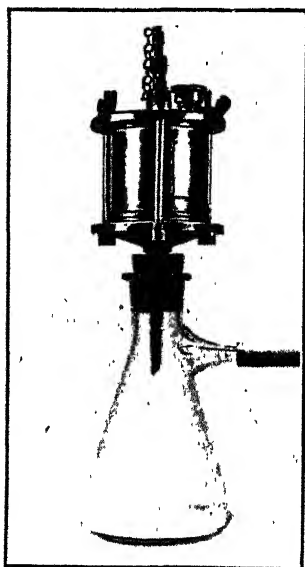


FIG. 21.—SEITZ FILTER.

laboratory use without resorting to heat. It is also used to separate soluble bacterial products, present in a fluid culture, from the bacteria themselves. The filters may be composed either of unglazed porcelain (Chamberland and Doulton), of diatomaceous earth (Berkefeld and Mandler), or of asbestos (Seitz). They are usually made in the form of thick-walled tubes, called candles, closed at one end and with the other end fitted with some kind of attachment (metal in the case of the Berkefeld and glazed porcelain in the Chamberland and Doulton filters), to which a rubber tube or stopper may be fitted.

Since filtration without the application of pressure would be too slow to be practicable, it is necessary to force the fluid through by applying pressure greater than that of the atmosphere or, as is more usually done, to utilize atmospheric

pressure by exhausting the receiving vessel, which may be accomplished with a Hyvac or other type of vacuum pump. Excessive pressure is to be avoided, as it may force bacteria through the pores of the filter and, on the other hand, filtration should be carried out as rapidly as possible, since bacteria may grow through even some of the finest filters if sufficient time is allowed. One method of using a small Chamberland

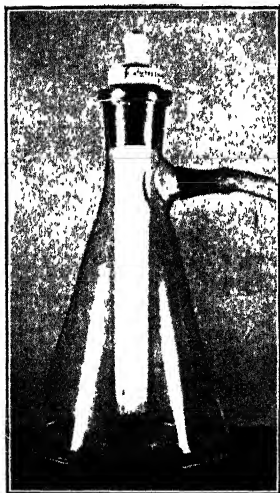


FIG. 22.—SMALL CHAMBERLAND FILTER, ARRANGED TO FILTER FROM WITHIN OUTWARDS.

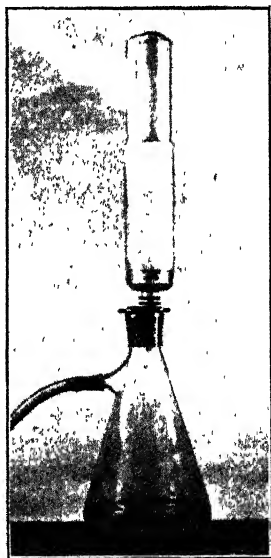


FIG. 23. — BERKEFELD FILTER ARRANGED TO FILTER FROM WITHOUT INWARDS.

filter is shown in Fig. 22. Here the fluid to be filtered is introduced into the candle, through the opening in the top, by means of a pipette, and the filtrate is collected in the flask, filtration being effected from within outwards. In Fig. 23 a Berkefeld filter is shown arranged to filter from without inwards. The candle is surrounded by a glass mantle, open at the top, into which the fluid to be filtered is poured. In

both cases a negative pressure is created in the receiving flask by means of a vacuum pump.

The Seitz filter is of quite a different construction. It is made of metal and allows a filtration disc of asbestos to be clamped in position. Fluid which escapes from the delivery tube below must have passed through this disc. In the type illustrated (Fig. 21), the receptacle is filled with fluid which may be forced through the disc either by atmospheric pressure, the air in the receiving flask having been exhausted by a vacuum pump, or by applying positive pressure to the receptacle by means of a bicycle pump attached to a valve situated on top.

Different grades of filters may be obtained, the coarser of which (Chamberland L1 and L2, Berkefeld V and Seitz K) cannot be relied on to prevent the passage of bacteria. None of the ordinary bacteria will pass through Chamberland L3, L5, or F, Doulton, Berkefeld N or W, or Seitz EK filters, and few of the filterable viruses will pass through Chamberland B, L7, L9, or L11 filters.

In all cases it is necessary to sterilize the assembled apparatus in the autoclave. When a candle has been used, its pores become choked and the filter works more slowly. The following method of cleaning can be recommended. The filter is soaked in bleaching lime solution to disinfect it. The outside is brushed with a stiff brush and is washed by aspirating or forcing saline through the wall in the reverse direction from that in which filtration proceeded. The candle is then boiled in 2 per cent. sodium carbonate solution for half an hour and then in several changes of water. Finally distilled water is again forced through the walls. All filters, whether new or cleaned, before being assembled should be tested for the presence of minute cracks. The simplest method is to immerse the candle under water and pump air into it by means of a rubber tube attached to it. If a crack exists, air will pass through it and the bubbles will be visible under the water.

Since a new disc is used in the Seitz filter for each filtration the question of cleaning does not arise.

### Animal Inoculation

Experiments on living animals are essential in bacteriology, but must not be performed in Great Britain or Ireland without a Government licence.

The common laboratory animals are guinea-pigs, rabbits, mice and rats. Material containing bacteria may be injected subcutaneously, intraperitoneally or intravenously. Less commonly other routes, into the anterior chamber of the eye, the heart or lung, may be employed. Subcutaneous injections in guinea-pigs and rabbits are best made by shaving the abdominal skin, treating with tincture of iodine and using a fine needle, inserted obliquely so as to avoid penetrating the abdominal wall. In the mouse or rat the skin near the root of the tail is most convenient. In intraperitoneal injection, after preparation of the skin of the abdomen, the whole thickness being pinched up between the finger and thumb, the needle is inserted into the midst of the fold thus formed, so avoiding penetration of the gut. When the fold is released, the needle will be in the peritoneal cavity. In the rabbit the marginal vein of the ear is used for intravenous injection. The covering skin is shaved and the vein distended by light pressure on it near the root of the ear. A fine needle is then introduced and the injection slowly given. If a swelling forms, the fluid is in the subcutaneous tissues and not in the vein. Intravenous injection of the guinea-pig is difficult, but intracardial injection can be carried out quite easily. Injections can be made into one of the veins situated near the root of a rat's tail.

Blood samples may be required from the living animal. In the case of the guinea-pig, the animal is anæsthetized with ether (a cone made from cardboard to fit over the nose and mouth and containing cotton wool moistened with ether is an excellent mask) and tied down on an operation board. A needle, similar to that used for human intravenous injections, is connected by a short rubber tube with a wide glass bulb to the other end of which a long rubber tube is connected. The apex beat of the heart is found and the needle inserted



in a backward and inward direction. Suction is applied by holding the end of the rubber tube in the mouth, and blood usually appears in the glass tube or, if not, the needle may be slowly withdrawn, suction being maintained, as sometimes the needle penetrates the heart completely. In this way about 10 c.cs. of blood may be withdrawn without endangering the animal's life.

Blood may be obtained from a rabbit's ear by puncturing the marginal vein with a triangular needle and allowing the blood to drop into a tube. If the ear is kept warm and the skin previously rubbed with vaseline about 50 to 60 c.cs. of blood may be taken from a suitable rabbit. If a rabbit is to be bled to death, it is first anaesthetized and the blood collected from the carotid artery after the vessel has been exposed, or blood is aspirated from the heart as described above in the case of the guinea-pig. A small blood sample may be obtained from the mouse or rat by cutting off the terminal portion of the tail.

An animal dying after inoculation should be thoroughly examined. It is tied on a board and the hair soaked with 2 per cent. lysol. The skin is completely reflected from the anterior of the thorax and abdomen; with fresh sterile instruments these cavities are opened and the organs required for culture are removed and placed in sterile Petri dishes. In many cases it is necessary to obtain a sample of the blood from the heart. This is done by searing the anterior surface of the organ with a cautery and penetrating this portion with a sharp sterile capillary pipette into which a few drops of blood are aspirated. Juice from other organs, for culture, may be obtained in a similar manner.

Rabbits are commonly employed for the preparation of antisera (bacterial agglutinating sera and hæmolytic sera for example). Various methods of inoculation are used, but we prefer the intravenous one. In this, injections are given from a syringe with a fine, sharp needle into the marginal vein of the ear. The skin is shaved and the vein distended by light pressure on it near the root of the ear before the needle is introduced. The dose varies with the material used. For the

enteric group of bacilli, the growth from one agar slope is either suspended in 10 c.cs. of carbol-saline and left at air temperature for a few days or in 10 c.cs. of saline and kept at 60° C. for 30 minutes. Either method is sufficient to kill the bacilli. The first injection consists of 1 c.c. of a  $\frac{1}{3}$  dilution of this suspension in saline, the second of 1 c.c. of a  $\frac{1}{4}$  dilution, the third of the same amount of a  $\frac{1}{2}$  dilution, and so on. Although phenol is somewhat destructive of O-type and heat of H-type antigens, the serum from an animal so treated should contain, if the bacteria were motile, both H and O agglutinins. If only O agglutinins are required the bacteria, previous to injection, may be treated with alcohol as described on p. 87. For the preparation of hæmolytic sera, the red cells are thoroughly washed in saline and a suspension in saline made of such a strength that, when mixed with an equal volume of water, the strength of the resulting solution of hæmoglobin is the same as that of Haldane's standard. The first injection consists of 1 c.c. of this suspension. The second injection is the same volume of double this strength, the third of four times, and so on. Different workers prefer different methods of spacing the injections. In our practice injections are given once a week for four or more weeks. A sample of blood is taken for testing one week after the last inoculation and, if considered satisfactory, the animal is bled either from the heart or from the carotid artery. The blood is allowed to clot and the clear serum pipetted off, any remaining cells being deposited either by gravity or centrifuging. The serum is kept in phials, but it is usual to add an antiseptic to prevent contamination. A few drops of chloroform, 0.2 per cent. of cresol, or an equal volume of glycerol may be used. We prefer to dilute agglutinating and hæmolytic sera with carbol-saline (one part serum to four parts carbol-saline) as we find in this way the titre of the serum is well preserved, no deposit forms and no contaminations occur.

### Preparation of Vaccines

Vaccines, as now employed either prophylactically or therapeutically, are suspensions of killed bacteria. The required

organism is grown on the surface of solid medium—agar or blood-agar most commonly—and the growth is washed off with sterile carbol-saline. The suspension is shaken with glass beads for some time, in order to break up clumps of bacteria, and is then left at rest for an hour to allow any large masses to fall to the bottom of the tube, when the upper part is pipetted off into a fresh tube and heated in a water-bath at 60° C. for one hour. This is sufficient to kill the majority of non-sporing bacteria. Vaccines of sporing organisms are rarely employed and, when such are required, special methods must be used. After heating, the sterility is tested by incubating a tube of broth or other suitable fluid medium inoculated with two or three loopfuls of the suspension. Owing to the presence of phenol, such test cultures cannot be made on solid medium.

The most satisfactory method of counting bacteria in a vaccine is to use a special chamber similar to a hæmocytometer with Thoma ruling, but with the cell only 0.01 mm. in depth. The suspension is accurately diluted to  $\frac{1}{100}$  or  $\frac{1}{1000}$ . A loopful of the dilution is placed on the ruled area, and the cover glass adjusted. The slide is then left for a few minutes to allow the bacteria to settle, and the preparation is examined microscopically. Two methods are available. The better is to use a dark ground condenser which renders the bacteria easily visible. Failing this, the dilution fluid should contain a small amount of methyl violet, and ordinary illumination should be used. The average number of bacteria seen per small square is then estimated. Each small square measures  $\frac{1}{20}$  mm. by  $\frac{1}{20}$  mm., and the depth is  $\frac{1}{100}$  mm. If we suppose that the average number of bacteria per square is 8 and the dilution of the suspension was  $\frac{1}{100}$ , the number of bacteria in the original suspension was  $8 \times 100 \times 20 \times 20 \times 100 = 32,000,000$  per cub. mm., or 32,000 million per c.c.

Since the action of vaccines on the patient is very much more variable than is that of drugs, we are doubtful if great refinements of the counting methods are of much value. We cannot argue that because a dose of 200 millions of a staphylococcus produced no reaction in one patient the result of that dose will be the same in another. In treat-

ment it seems best to commence with a dose almost certainly too small and to increase this subsequently until we reach the level of therapeutic activity. The method which we employ in standardizing a vaccine is to dilute until the strength, as judged by comparison with previously prepared standard opacity tubes, such as those of Brown, is 5,000 or 10,000 million per cubic centimetre. Different organisms in suspensions of the same concentration have different degrees of opacity, and it is necessary to have at hand standards for different organisms—staphylococci, gonococci, *Bact. coli*, and others. By knowing the dilution, the strength of the original suspension can easily be estimated.

Vaccines are diluted for use and are supplied either in sealed glass phials, each intended for a single dose, or in rubber-capped bottles containing 10 to 20 c.cs., for repeated use. The latter are much more easily prepared and are more convenient and economical in use. The bottles containing 10 c.cs. of carbol-saline are autoclaved. The rubber caps are boiled in 5 per cent. carbolic acid and are fitted on the bottles as soon as these are removed from the autoclave. The final dilutions should always be made in carbol-saline. Supposing we have a suspension of 10,000 million staphylococci, and require to make a vaccine in two strengths—one of 200 million per cubic centimetre (A) and the other of 1,000 million per cubic centimetre (B). Two 10 c.c. bottles are taken and their rubber caps treated with a disinfectant (5 per cent. carbolic or 2 per cent. lysol). A 1 c.c. Record syringe is sterilized by boiling. The top of A is punctured with the needle and 0.20 c.c. of saline removed. In the same way 1.0 c.c. is removed from B. To A 0.20 c.c. of the stock vaccine (*i.e.* 2,000 million bacteria) is added, giving a strength of 200 million per cubic centimetre, and 1.0 c.c. to B, giving a strength of 1,000 million per cubic centimetre. The caps are then wired on and the bottles labelled. When a dose of the vaccine is required the cap is treated with disinfectant, after shaking the bottle, and the necessary amount taken up into a sterile 1 c.c. syringe for injection.

Injectons are usually given subcutaneously at intervals

of five to seven days, which period avoids any risk of re-injection during the negative phase. The response we hope for is a specific one, and for that reason we use an autogenous vaccine, prepared from the bacteria isolated from the patient, in preference to a stock vaccine, prepared from the same organism, but from a different patient, or usually a mixture of the organisms of several patients. In addition to the specific response there appears also to be a non-specific response to the injection of almost any vaccine, since a few hours after an injection of, say, a staphylococcus vaccine, the patient has developed anti-substances capable of dealing with streptococci and other types of bacteria. It is probably this non-specific response which explains the good results occasionally obtained in certain acute conditions, such as puerperal sepsis, by the frequent injection, daily or on alternate days, of a vaccine in small doses.

It happens, not uncommonly, that good results are obtained by the use of a vaccine where such results were scarcely to be expected. These are probably to be attributed to a non-specific response. This is a perfectly legitimate use for vaccine therapy, but it is quite illegitimate to deduce, as is sometimes done, that because cure follows the use of a vaccine the organism employed in the preparation of the vaccine, is identical with that causing the disease. Good results were reported by using typhoid vaccine in the treatment of typhoid fever, but equally good results were obtained with *Bact. coli* vaccines. Nor does a focal reaction prove any connection between the organism of the vaccine and the disease. Administration of sanocrysin in tuberculosis provokes focal reactions similar to those produced by tuberculin.

Wright has advocated the intravenous administration of vaccines in acute infections, since it is possible to demonstrate *in vitro* an increase in the bactericidal power of blood when a vaccine is added to it. This response, which is non-specific, is probably due to the extrusion of opsonic and bactericidal substances from the leucocytes.

It is difficult to speak very definitely of dosage. It is best to start with a very small dose, and to increase rapidly

until a slight reaction is obtained (headache, malaise, local pain, or a slight rise in the temperature) ; then the steps must be made smaller. All severe reactions are to be avoided. It is suggested that the initial dose of autogenous vaccines of *Streptococcus pyogenes* should be from 2 to 10 millions, of pneumococci 10 millions, of gonococci, *Bact. coli*, *Proteus vulgaris*, *N. catarrhalis* and diphtheroid bacilli 20 millions and of staphylococci 50 millions. When stock vaccines are used the dose may be two or three times as large as these, and for prophylaxis they may be about five times as large. It is convenient to prepare a vaccine of such a strength that the first dose will be contained in 0.1 c.c.

A mixed vaccine is one containing more than one variety of organism. It is used in conditions where more than one type of bacterium is believed to be causative ; in acne, for example, where both the *C. acnes* and a staphylococcus are at work, and also in chronic bronchitis, where it may be impossible to say which of the several predominant organisms are of chief importance, or whether each may be pathogenic.

Vaccines have their greatest use in sub-acute and chronic bacterial infections, particularly in those due to the staphylococci, *Bact. coli* and gonococci, and also in prophylaxis (of the enteric fevers, for example) and are only to be used with caution in very acute infections.

In addition to the vaccines mentioned above many other types have been introduced. Detoxicated and residual vaccines, it is claimed, contain the nitrogenous substances of bacteria freed from endotoxin. They are prepared either by dissolving the bacteria in caustic soda solution and precipitating with acid, by treatment with other chemicals or by grinding the bacteria in a mill. Enormous doses can be given as these vaccines have very slight toxicity, and some workers claim good results from their use.

Sensitized vaccines (Besredka) are prepared by treating the bacteria with antiserum, the former being subsequently freed from the latter by centrifuging and washing. The bacterial antigens probably combine with antibodies of the serum. These vaccines appear to be particularly successful

in the treatment of acute conditions. It is claimed for them that immunity is rapidly developed, is of long duration, and that less toxic effects are experienced than with ordinary vaccines.

Besredka introduced the oral method of administration of vaccines. If a bile pill is previously swallowed, the bile appears to allow of the absorption of the bacteria by the intestinal mucous membrane. Besredka claims that a local immunity of the intestinal wall is so developed, but this is very doubtful. The method is chiefly used in the prophylaxis of the intestinal diseases, and moderately good results are stated to have been obtained.

Besredka has also introduced "antiviruses," which are broth cultures filtered to remove bacteria and heated to reduce toxicity. He claims that a staphylococcal antiviral applied to the skin as a wet dressing, produces local immunity against staphylococcal infections, but it has been found that plain broth, peptone solution and mustard plaster act in the same way. The immunity, which is only local, is non-specific and is probably due to a local cellular reaction and to hyperæmia. Antiviruses, prepared against organisms causing respiratory infections, are used as a spray in the hopes of conferring immunity against these bacteria.

## CHAPTER VII

### SEROLOGICAL TECHNIQUE

This chapter, which deals with the practical aspects of serology, should be read in conjunction with the theoretical consideration of the subject given in Chapters XV, XVII, and XVIII.

#### Agglutination Tests

THE agglutination of bacteria by antisera may be employed in the laboratory either :—

- (1) To assist in the diagnosis of disease. In this case the serum of the patient is mixed with a suspension of known bacteria.
- (2) To identify an organism. Here the serum of an animal, immunized with a known organism, is mixed with the unknown bacteria.

There are three methods of carrying out agglutination tests :—

- A. Slide method.
- B. Microscopic method.
- C. Macroscopic method.

In the following pages questions of dilution are frequently mentioned. When a dilution of serum in saline, for example, is stated to be  $\frac{1}{5}$ , this means that one part of serum is present in every five parts of the diluted preparation. 1 part serum + 4 parts saline = 5 parts of  $\frac{1}{5}$  dilution.

**A. The Slide Method.**—Suppose we have a culture of an organism which we believe to be *Bact. typhosum*. Take an ordinary microscope slide and with a platinum loop place, near one end, a drop of anti-typhoid serum either undiluted or only slightly diluted ( $\frac{1}{5}$  or  $\frac{1}{10}$ ) and near the other a drop of saline. With the loop, take up some of the culture and



emulsify it in each drop, so as to form suspensions which are much denser than those made in preparing films for staining. Rock the slide for a few seconds; if the organism is the typhoid bacillus, the suspension in the drop of serum will become lumpy and, in less than a minute, small floccules of agglutinated bacilli, visible to the naked eye, will be seen floating in a clear liquid; the suspension in the other drop will remain uniformly turbid. This method is not to be taken as a conclusive proof of the identity of the organism, but merely as a valuable indication. It is used chiefly in examining the colonies on a plate in order to determine which should be subcultured for further investigation. If the plate was



FIG. 24.—SLIDE AGGLUTINATION.

A culture of *Bact. typhosum* was mixed with drops of anti-sera prepared against *Bact. typhosum* (T), *Bact. paratyphosum* A (A), and *Bact. paratyphosum* B (B). Agglutination has occurred with T, and not with A or B.

made from a stool of a case of suspected enteric fever, it is convenient to arrange four drops on the slide :—(1) anti-typhoid serum, (2) anti-paratyphoid A serum, (3) anti-paratyphoid B serum, and (4) saline. The last of these is a control to detect those organisms which agglutinate spontaneously. If agglutination occurs in the third drop and not in the others, it suggests that the organism in the colony is the *Bact. paratyphosum* B. In the same way anti-Shiga serum and anti-Flexner serum may be used in investigating a case of dysentery. The fallacies of the method are that, using strong serum, agglutination may occur with organisms other than those used in the immunization of the animal from which the agglutinating serum was obtained, and also that

a pathogenic organism freshly isolated from the body may be relatively inagglutinable.

**B. Microscopic Method.**—This may be used for either (1) or (2) above. When used for (1) (diagnosis of disease) it constitutes the Widal reaction. A living broth culture of the organism (say *Bact. typhosum*), about eighteen hours old, is used. Three dilutions of the patient's serum in saline are required ( $\frac{1}{12}$ ,  $\frac{1}{24}$  and  $\frac{1}{48}$ ). These may be made either by a dropping method as in Dreyer's technique (p. 84), or by drawing a volume of serum, followed by the necessary number of equal volumes of saline, separated from one another by air bubbles, into a capillary pipette (p. 90). A loopful of the  $\frac{1}{12}$  dilution is placed on a cover slip, and to it is added, with the same loop, a similar amount of the broth culture and the two are mixed. From this a hanging drop preparation is made. Similar preparations are made with the  $\frac{1}{24}$  and  $\frac{1}{48}$  dilutions. Since, in each case, a drop of the dilution is mixed with an equal volume of the broth culture, the serum will be present in the three microscopical preparations in dilutions of  $\frac{1}{24}$ ,  $\frac{1}{48}$ , and  $\frac{1}{96}$  respectively, or approximately  $\frac{1}{25}$ ,  $\frac{1}{50}$ , and  $\frac{1}{100}$ . In every case it is necessary also to prepare a control with the broth culture and saline only. It is best to put the preparations in the incubator for twenty minutes and then examine. In the control the bacilli will be found to be free from one another, evenly distributed and actively motile. If the patient has suffered from typhoid fever of more than two weeks' duration, in the first certainly, the second probably, and the third possibly, agglutination will have occurred. This is recognized by the fact that the bacilli are no longer motile, or at most exhibit only slight vibratory movements, and are no longer evenly distributed, but are collected in a few large clusters. Where Widal's reaction is used in diagnosis it is advisable to put up the serum not only against the typhoid bacillus but also against the two paratyphoid bacilli. The test may be used in other diseases, but is of greatest value in the enteric fevers. The interpretation of the results of Widal tests is considered in the chapters dealing with typhoid and paratyphoid fevers.

**C. Macroscopic (Dreyer's) Method.**—By using this method accurate results may be obtained which are exactly comparable with those of other observers. In using it for a Widal reaction the suspension of bacteria is obtained by killing the bacteria in a broth culture with formalin (0.1 per cent.) and diluting with water till of suitable opacity (500 to 1,000 million per c.c.): the suspension of dead bacteria keeps well. In testing the serum of a patient for the presence of agglutinins (of H type) against the bacilli of the

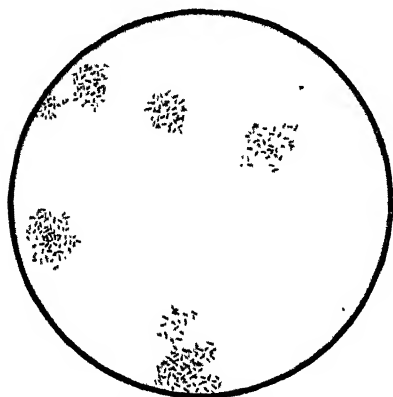


FIG. 25.—MICROSCOPIC AGGLUTINATION.

Complete agglutination obtained by mixing a broth culture of *Bact. typhosum* with the serum of a typhoid patient ( $\times 200$ ).

enteric group the procedure is as follows: A small metal rack is taken, provided with three rows of five small holes and four larger holes, two at either end. In the small holes are placed the special agglutination tubes with pointed ends and, in the larger, tubes measuring 9 cms. by 1 cm. With the special standard pipette, filled with saline and held vertically, drops are counted as follows: into one large

tube 54 drops, into the first small tube in each row 0, into the second 5, into the third 8, into the fourth 9, and into the fifth 10. The serum is next taken up and 6 drops are added to the large tube and the contents mixed after the pipette has been washed; this gives a  $\frac{1}{10}$  dilution of serum. The pipette is well rinsed or, if very accurate work is attempted, is washed with water followed by alcohol and then ether and finally air is drawn through to dry it. This procedure should strictly be adopted with each change of reagent, but for clinical work it is sufficient to wash well and to expel as much moisture as possible. The diluted

serum is taken up and added to the tubes of each row—first 10 drops, second 5, third 2, fourth 1, fifth 0. To all the tubes in the front row 15 drops of the typhoid suspension are added, a like quantity of the paratyphoid A suspension to the tubes of the middle row and of the paratyphoid B suspension to the back row. The contents of the tubes are shaken by holding the finger over the end and inverting each tube in turn, the finger being wiped between each. The rack is

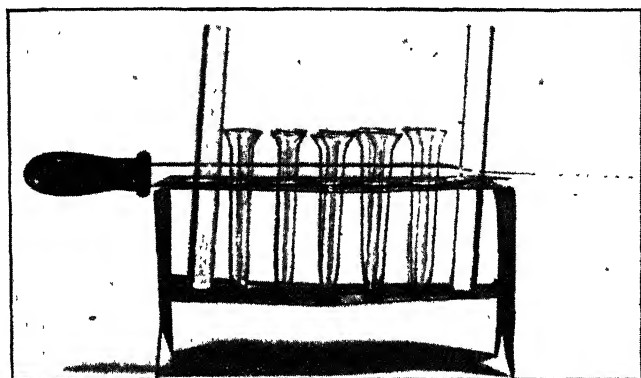


FIG. 26.—OUTFIT FOR PERFORMING MACROSCOPIC AGGLUTINATION TESTS BY DREYER'S METHOD.

incubated in a water-bath at 52° C., so arranged that the level of the water is about one-third way up the column of fluid in the tubes. The system of dilution may be made clearer as follows, taking the front row of tubes only:—

	Tube 1.	2.	3.	4.	5.
Saline ..	0	5	8	9	10
Serum ( $\frac{1}{10}$ )	10	5	2	1	0
T. suspen- sion ..	15	15	15	15	15

Since the total volume in each tube is 25 drops, the concentration of serum in the first tube is  $\frac{10}{25}$  of  $\frac{1}{10} = \frac{1}{25}$ . Similarly it is found that the serum is diluted  $\frac{1}{50}$  in the second,  $\frac{1}{125}$  in

the third and  $\frac{1}{250}$  in the fourth: the fifth acts as a control. In the middle row the serum acts in similar concentrations on *Bact. paratyphosum* A and in the back row on *Bact. paratyphosum* B.

After two hours' incubation the rack should be removed from the bath, left for ten minutes at room temperature and examined. The results are most easily seen if the tubes are held against a black background and illuminated from above and behind. In the case of typhoid fever the first and second tubes in the front row may show complete agglutination and sedimentation. The bacilli are collected in a

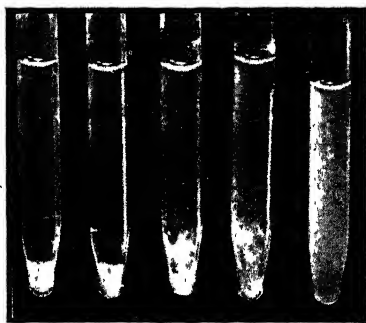


FIG. 27.—A POSITIVE MACROSCOPIC  
AGGLUTINATION TEST.

Complete agglutination is present in the first two tubes, partial in the third and fourth. The fifth tube is a control and shows no agglutination.

mass at the bottom of the tube, leaving the upper part clear. In the third there may be marked agglutination with some sedimentation and in the fourth standard agglutination, all the bacilli being collected in fine floccules just visible to the naked eye, but without sedimentation. In the fifth there should be a uniformly turbid suspension. The result in this case might be reported as standard agglutination  $\frac{1}{250}$ .

In order to obtain comparable results at different times and in different places, it is advisable to employ "standardized suspensions," such as may be obtained from the Standards Laboratory of the Medical Research Council at Oxford.

These are of correct density and are compared, as regards agglutinability, with an arbitrary standard suspension. When the agglutinability of one of these is significantly different from that of the standard, it is assigned a suspension factor, by the use of which such difference may be corrected. The standard titre of a serum is calculated by dividing the denominator of the observed titre by the factor. If, with a suspension the factor of which is 0.5, the recorded titre is  $\frac{1}{250}$ , the standard titre is  $\frac{1}{250 \div 0.5} = \frac{1}{500}$ .

In the same way we determine the result if one of the other organisms is agglutinated but not the *Bact. typhosum*. Where all the tubes in a row which contain serum (1 to 4) show agglutination, it is necessary to make a further dilution and set up another series of tubes. This is done by adding 3 drops of the  $\frac{1}{10}$  dilution to 57 of saline and using this ( $\frac{1}{200}$ ), as before, giving in the tubes of the second series dilutions of  $\frac{1}{500}$ ,  $\frac{1}{1000}$ ,  $\frac{1}{2500}$ ,  $\frac{1}{5000}$ . For diagnosis in the uninoculated and in those who have not previously suffered from one of the fevers, a standard titre of  $\frac{1}{50}$  is almost diagnostic, although much higher titres are usually obtained. In the inoculated a certain amount of information may be obtained by making tests at intervals of a few days. Considerable alterations (first rising and, later, falling) in the titre indicates infection with the agglutinated organism. Similar alterations, although less marked, also occur with related organisms (the paratyphoids in the case of a typhoid infection, for example).

The serological diagnosis of enteric infections is rendered more reliable if O type as well as H type agglutination is employed. A dense suspension of the bacilli from agar is made, and to it is added an equal volume of absolute alcohol. The mixture is kept for twenty-four hours at 37° C., so killing the bacilli and destroying the H agglutinin, and to it is added one-half volume of saline. This suspension, when required for use, is diluted with water sufficiently to give a suspension of the correct density. The test is carried out exactly as before, using this suspension, except that incubation is prolonged for twenty to twenty-four hours at 52° C. In

reading the results a good lens must be employed, since agglutination occurs in fine granules. Even in the inoculated a titre of  $\frac{1}{100}$  by this method is almost diagnostic. Where non-motile bacilli (such as the dysentery bacilli) are employed only O type agglutination occurs. A suspension of the bacilli, killed by heat at 60° C. for one hour or by formalin, is used and the tubes are incubated at 52° C. for twenty to twenty-four hours.

It may occasionally be necessary to discover whether the serum of a patient or suspected carrier contains Vi agglutinin. This may be done by using either a living suspension or one which has been treated with formalin (0.6 per cent.) or mercuric chloride (1 in 1,000) of a strain of *Bact. typhosum* which is non-motile (that is, has lost H antigen) but contains sufficient Vi antigen to prevent its agglutination by O agglutinin. Incubation should be at 37° C. for four hours. If the serum agglutinates the suspension, it contains Vi agglutinin. Vi variants of *Bact. typhosum* have been isolated which are entirely devoid of O antigen. These may be used as pure reagents for the detection of Vi agglutinin.

The method is also applicable to the reverse operation (identification of an unknown organism with a known serum). Owing to the higher titre of the serum of an immunized animal than of a human patient, it is usually necessary to proceed to the second series of tubes, reaching a dilution of  $\frac{1}{5000}$ , or even a third (using  $\frac{1}{4000}$  serum and reaching in the last tube  $\frac{1}{100000}$ ).

An organism, such as the typhoid bacillus, when freshly isolated from the body, may have temporarily lost its flagella and so may not be agglutinated by a serum containing H. agglutinins. Culture for several generations on artificial media usually leads to a return of flagella production. A freshly isolated *Bact. typhosum* may also fail to be agglutinated by a serum containing O agglutinins owing to the presence of Vi antigen. Such cultures, in the living condition, are readily agglutinated by a Vi immune serum or, when killed by heat or alcohol, which destroy Vi antigen, by O immune serum.

No agglutination test should be recorded as negative after a failure to detect agglutination in the first tube of the series, since it is occasionally found that earlier tubes show no agglutination while, in later tubes, agglutination is unmistakable. This is the pro-zone or zone of inhibition phenomenon.

### Absorption of Agglutinin

Simple agglutination carried to its end point is generally sufficient to identify an organism quite definitely. In the case of a few closely related organisms, however, the serum of an animal, immunized with one, may agglutinate one of the other members of the group to the same or even to a higher titre than it does its homologous organism. If the serum is saturated with its homologous organism, all agglutinins will be absorbed and the serum will no longer agglutinate either. If saturated with the related organism it will lose its power of agglutinating this, but will still agglutinate the homologous organism. Saturation may be effected by mixing 0.5 c.c. of the serum with about 5.0 c.c.s. of a very dense suspension of the organism, obtained by washing off the growth from about half a dozen agar slopes in a few cubic centimetres of saline. The mixture is heated to 50° C. for one hour and then, after again mixing, is left overnight at room temperature. It is thoroughly centrifuged to deposit all bacteria and the clear fluid pipetted off. The deposited bacteria are mixed with a few cubic centimetres of saline and again centrifuged. The washings are added to the first fraction and the volume made up to 10 c.c.s. This will give a  $\frac{1}{20}$  dilution of "absorbed" serum, which is then tested by the macroscopic method, against both the organism used for absorption, to see if absorption has been completed, and against the related bacterium. The following are the results of an experiment:—

Serum anti-paratyphoid B

Against Bact. paratyphosum B	—	Agglutination $\frac{1}{5000}$
Against Bact. typhi-murium	—	„ $\frac{1}{2500}$



Serum anti-paratyphoid B (after saturation with Bact. paratyphosum B)

Against Bact. paratyphosum B — No agglutination  $\frac{1}{50}$

Against Bact. typhi-murium — „ „ „ „

(Both specific and group agglutinins have been absorbed.)

Serum anti-paratyphoid B (after saturation with Bact. typhi-murium)

Against Bact. paratyphosum B — Agglutination  $\frac{1}{2500}$

Against Bact. typhi-murium — No agglutination  $\frac{1}{50}$

(Group agglutinins absorbed, specific agglutinins not absorbed.)

If an unknown organism (X) is able to remove all the specific (P) agglutinins from an anti-P serum, it is practically certain that P and X are identical. The identity is conclusively proved if it is also found that P will remove all specific (X) agglutinins from a serum prepared against the unknown organism X.

### Opsonic Methods

The phagocytic action of the polymorphonuclear leucocytes may be very simply demonstrated thus. Take a capillary pipette with a mark about 2 cms. from the tip. A loopful of a young culture of a staphylococcus is rubbed up thoroughly in a few cubic centimetres of saline, and this is diluted until a suspension which is only just opalescent is obtained. A volume of citrate solution is drawn up into the pipette, then an air-bubble, then a volume of blood obtained by pricking the finger and, after another air-bubble, a volume of the suspension of cocci. The whole is discharged on to a slide and mixed by drawing up and expelling two or three times. It is finally drawn up some centimetres from the tip, which is sealed in a small flame. The pipette is incubated at 37° C. for a quarter of an hour, when the tip is broken off, the contents mixed as before, and films prepared in the same way as are ordinary blood films. These are stained with Leishman's stain and examined. Within the majority of the leucocytes will be found ingested cocci, in some only one or two, in others large numbers. By finding the average number per leucocyte we can measure the phagocytic power of the

blood. This depends chiefly, not on the leucocytes themselves, but on the presence of opsonins in the serum, and hence it is more usual to measure, not the phagocytic power of the leucocytes, but the opsonic power of the serum.

**Determination of Opsonic Index.**—(1) A suspension of the organism is obtained as described above. It is essential that the bacteria in this should be isolated from one another and that no clumps should be present. To secure this, it may be necessary to shake the suspension with glass beads and to centrifuge in order to deposit the larger masses. If it is thought desirable, the bacteria may be killed by heat or by the addition of formalin, which must be eliminated by centrifuging, washing in saline and further centrifuging, before the suspension is prepared.

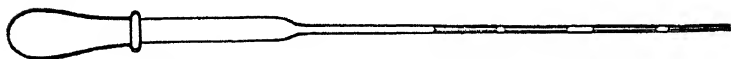


FIG. 28.—METHOD OF USING A CAPILLARY PIPETTE FOR MEASURING EQUAL VOLUMES OF FLUIDS, AS IN THE DETERMINATION OF THE OPSONIC INDEX.

(2) *Patient's serum.*—Sufficient blood may be obtained from a finger. It is collected in a small tube and, after clotting has occurred, the serum is separated by centrifuging: it must be freshly obtained.

(3) *Control serum.*—Procured in the same way as the above from a healthy person, or better, the mixed sera of a number of healthy persons may be used.

(4) *Blood cells.*—About 10 drops of blood from the finger of the observer (preferably of blood group O) are dropped into a centrifuge tube containing sodium citrate solution. After mixing, this is centrifuged, the fluid removed, saline added and re-centrifuged. The citrate prevents clotting, and both it and the saline wash the cells free from serum. The deposited cells are thoroughly mixed with fresh saline.

In a pipette, by the method described for determining the phagocytic power of the blood, a mixture of equal parts of (1), (2) and (4) is obtained, and in the same way, in another pipette, a mixture of (1), (3) and (4). The pipettes are incubated for

fifteen minutes at 37° C. and, after mixing, films are prepared from each and stained by Leishman's method (if tubercle bacilli are used, the Ziehl-Neelsen method must be employed). The number of bacteria ingested by 100 polymorphonuclear leucocytes in each case is determined, and from this the average per leucocyte is found. The two preparations are exactly similar, except that in the first the leucocytes and bacteria were in contact with the patient's serum, and in the second they were acted upon by normal serum. The opsonic index is: (Average number of bacteria per leucocyte in the preparation with patient's serum)  $\div$  (Average number of bacteria per leucocyte in the preparation with normal serum).

The normal index is of course 1, and it is exceptional to find a figure lower than 0.5 or higher than 2.

### **Determination of the Bactericidal Power of Serum**

The bactericidal power of a serum may be demonstrated by mixing various dilutions of it with constant amounts of a suspension of bacteria and incubating for a few hours. Cultures are prepared from the mixtures and it is found that in the earlier tubes the organisms are killed but not in those containing serum diluted more than  $\frac{1}{100}$ . This is due to the fact that the serum is deficient, not in antibodies, but in complement. It is therefore better to use various dilutions of the serum, previously heated to 55° C., and to add to each tube a constant amount of the fresh serum of a normal animal to supply complement. In this way it is found that the presence of complement, supplied by normal guinea-pig serum, increases the killing titre of the immune serum. In such an experiment the Neisser and Wechsberg phenomenon is sometimes observed. This is the absence of killing in the tubes containing the largest amount of immune serum, while the bacteria are all destroyed in the presence of smaller amounts.

### **Hæmolytic Tests**

The principal facts concerning the mode of action of hæmolytic sera can be very simply demonstrated.

(1) Collect some blood from a sheep into citrate solution

to prevent clotting. Concentrate the corpuscles by centrifuging and wash in several changes of saline, centrifuging the cells free from the saline each time. Re-suspend the cells in saline, making about a 2 per cent. suspension.

(2) Collect some blood from a rabbit which has received, on two or three occasions, intravenous injections of a suspension of washed sheep cells. Allow to clot and separate the serum.

(3) Collect blood from a normal guinea-pig, allow to clot and separate the serum. (See pp. 49 and 50.)

Four small test tubes 9 cms.  $\times$  1 cm. are taken and the following reagents are added, measurements being made in cubic centimetres.

	1.	2.	3.	4.
Saline .. ..	1.0	1.0	1.0	0.5
Fresh serum of the immunized rabbit (diluted $\frac{1}{20}$ )	0.5	—	—	—
The same diluted serum heated to 55° C. for 10 minutes	—	0.5	—	0.5
Fresh serum of normal guinea-pig (diluted $\frac{1}{10}$ )	—	—	0.5	0.5
Suspension of sheep cells	0.5	0.5	0.5	0.5

The tubes are shaken and incubated in a water-bath at 37° C. for half an hour. The first and fourth will have transparent red fluids, showing that hæmolysis has occurred. The second and third will still be turbid and, if left for some time, the uninjured red cells will sink to the bottom of the tube, leaving the fluid above without any red colour. For hæmolysis it is necessary to have both hæmolysin (or immune body) and complement. Both are present in the fresh serum of the immunized animal. The complement is destroyed by heating to 55° C.; but it may be replaced by the addition of fresh normal serum, such as that of the guinea-pig.

Note that a temperature of 37° C. is used for all tests in

which either blood cells or complement are employed : higher temperatures damage both. Note also that normal saline (0.85 per cent. sodium chloride) is always used in the presence of red blood cells as these would be hæmolyzed by water.

### Wassermann Reaction

It may be well first to outline the principles on which this rather complicated test is based. In the blood serum of a syphilitic there are antibodies which act as if they were antibodies developed against such lipoidal substances as are found in an alcoholic extract of heart muscle. When these antibodies and their supposed antigens are brought into contact, complement, if present, is fixed as it always is when antigen and antibody interact. So we mix patient's serum, antigen (*i.e.* an alcoholic extract of heart muscle) and guinea-pig serum (containing complement). If the patient is syphilitic, the complement will be used up or fixed : if not, it will remain free as there is, in the mixture, no antibody to combine with the antigen. After a time we add red blood cells and the serum of an animal immunized against these cells and, therefore, containing hæmolysin. This acts as an indicator for complement. If complement is present, hæmolysis of the cells occurs. But, if complement is present at this stage, it was not fixed earlier. If it was not fixed earlier, this was because there was no syphilitic antibody in the patient's serum and, therefore, the result of the test is negative, and the patient is not syphilitic. If, on the other hand, the cells are not hæmolyzed, the test is positive and the patient is syphilitic.

There are almost innumerable methods of performing this test, but the one here given (which is based on Harrison's) is, in our opinion, one of the most reliable. The reagents required are :

(1) Suspension of washed sheep's red blood corpuscles—see above.

(2) Hæmolytic serum (immune body, I.B.)—see above.

(3) Fresh guinea-pig serum (complement)—see above.

(4) Antigen.—This is prepared as follows : Take a fresh, healthy human heart, free the muscle from fat, and grind

1.0 gm. with dry clean sand and 9 c.cs. of absolute alcohol. Let the mixture stand for  $1\frac{1}{2}$  hours, shaking occasionally; then filter through filter paper. This constitutes the heart extract. A solution of cholesterol (1 per cent.) in absolute alcohol is made. For use, 3 parts of the heart extract are mixed with 2 parts of the cholesterol solution in a large dry test tube or cylinder, and 70 parts of saline are rapidly added. The resulting turbid suspension is the antigen as used in the test.

(5) Patient's serum. After separation from the clot the clear serum is heated to  $55^{\circ}$  C. for half an hour to destroy complement. In addition, when a batch of tests is being done, it is advisable to re-test known positive and negative sera as controls.

So many reagents are employed in the test that it is necessary to standardize each as thoroughly as possible. The first to be considered is the blood suspension. Many workers are content to dilute the sedimented cells to a definite extent (*e.g.* to make a 5 per cent. suspension); but we prefer to standardize by the content of hæmoglobin, which may be compared with the standard tube of a Haldane's hæmoglobinometer. In our practice the suspension is of such a strength that when it is diluted  $\frac{1}{8}$  with distilled water and coal gas passed through, the resulting solution of carboxyhæmoglobin exactly matches the Haldane's tube.

**Titration of Immune Body.**—The immune rabbit's serum (I.B.), which has been heated to  $55^{\circ}$  C. for half an hour, is diluted, say,  $\frac{1}{1000}$ , and to a series of tubes are added the following reagents measured in cubic centimetres:—

	1.	2.	3.	4.	5.	6.	7.
Saline .. ..	0.0	0.25	0.5	0.6	0.7	0.8	0.85
I.B. ( $\frac{1}{1000}$ ) ..	1.0	0.75	0.5	0.4	0.3	0.2	0.15
Fresh guinea-pig serum (diluted $\frac{1}{10}$ ) ..	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Standard blood suspension (diluted $\frac{1}{2}$ with saline) ..	0.5	0.5	0.5	0.5	0.5	0.5	0.5

The contents of the tubes are mixed and the rack incubated at 37° C. for half an hour. If the hæmolytic serum is a good one it will probably be found that tubes 1 to 6 show complete hæmolysis (*i.e.* the fluid is quite clear without any turbidity), while tube 7 shows an opalescence which is due to unhæmolytised cells. The last tube showing complete hæmolysis contains one minimum hæmolytic dose (M.H.D.) of the immune body. In this case the M.H.D. is 0.2 c.c. of  $\frac{1}{1000}$  serum or 0.0002 c.c. of serum. In this titration it is to be noted that in each tube we had present 0.5 c.c. of a  $\frac{1}{2}$  dilution of the standard blood suspension which is equivalent to 0.25 c.c. of the standard suspension.

A dilution of the immune body is now made so that it contains 5 M.H.D. in every 0.25 c.c. In the titration recorded above 1 M.H.D. = 0.0002 c.c., and therefore 5 M.H.D. = 0.001 c.c. So we make a dilution of the I.B. containing 0.001 c.c. in a total volume of 0.25 c.c. or, since we shall require a larger volume, an equivalent dilution such as 0.2 c.c., in a total volume of 50 c.c., saline being used as the diluent. To this we add an equal volume of standard cell suspension, and this mixture constitutes the "Sensitized Cell Suspension" which, in each 0.5 c.c., contains the correct amount of cells and 5 M.H.D. of I.B. This mixture is an incomplete hæmolytic system, only requiring to be incubated at 37° C. with complement in order to produce complete hæmolysis of the red cells present. Immune body (hæmolytic serum) is a fairly stable substance, and it is not necessary to standardize it on each occasion when Wassermann tests are being performed. This must be done when a fresh batch of serum is brought into use, and subsequently every few months. We then note the M.H.D. of the serum, prepare a dilution such that 0.25 c.c. will contain 5 M.H.D., mix this with an equal volume of standard cell suspension, and so prepare the sensitized cell suspension, of which 0.5 c.c. is used in each tube in the subsequent steps.

**Titration of Complement.**—The fresh serum of a healthy guinea-pig (preferable male; but, if a female, not pregnant or recently delivered), bled either the same day or the previous





placed one behind the other. Into the three tubes the following reagents are measured, a fresh pipette being used for measuring each serum. It is usual to measure out the serum to be tested into the tubes first, then to add the necessary saline to all, and subsequently the complement and antigen.

	Front.	Middle.	Back.
Patient's serum } (heated to 55° C.) }	0·1	0·1	0·1
Saline .. .. .	0·4	0·4	0·9
Complement 3 M.H.D. ..	0·5	—	0·5
Complement 5 M.H.D. ..	—	0·5	—
Antigen .. .. .	0·5	0·5	—

The tubes are shaken, left at room temperature for half an hour and incubated in the water-bath at 37° C. for the same time. Then 0·5 c.c. of sensitized cell suspension is added to each; they are again mixed and incubated for half an hour. The back tube acts as a control, and in every case should show complete hæmolysis. If hæmolysis is not complete the patient's serum is "anti-complementary" and, unless the other two tubes of the serum show complete hæmolysis (negative reaction), the result must be discarded and a further sample of serum procured for testing. The front and middle tubes differ only in that the front contains 3 doses of complement and the middle 5. If the reaction of the serum be positive, the complement will have been fixed in the first stage of the test and the tubes will show no hæmolysis. If negative, the complement will have been left free and hæmolysis will be complete. In the case of a reaction weaker than full positive it may be found that the middle tube shows complete or partial hæmolysis, and the front, with only 3 M.H.D. of complement, no hæmolysis. Owing to the presence of the two tubes containing different strengths of complement we can determine, to some extent, the strength of the reaction. Various methods of recording the results are used. In the first, two symbols are employed, the first of which shows the result obtained in the tube containing 3 M.H.D. of complement and the second, in that containing 5 M.H.D. + signifies

a positive reaction (no hæmolysis),  $\pm$  or  $-$  a partial positive (partial hæmolysis), and  $-$  a negative reaction (complete hæmolysis). The second system is usually preferred by the clinician, while the third is that recommended by the League of Nations Health Committee.

First system.	Second system.	Third system.	Interpretation.
$++$	$+4$ }	$++$	Full positive
$+\pm$		$++$	Strong positive
$+ -$	$+3$	$+$	Positive
$\pm -$	$+2$ }	$\pm$	Weak positive
$\pm -$	$+1$ }		Doubtful
$--$	$0$	$-$	Negative

Various results obtained in the Wassermann reaction are illustrated in *Plate II*.

Other complement fixation tests such as those for gonorrhœa and tuberculosis, may be carried out in a similar fashion, the antigen usually being a suspension of the bacteria causative of the disease. In the case of echinococcus infestations a complement fixation test has been found of value.

### Kahn Reaction

A number of tests which have been introduced for the diagnosis of syphilis are based on the fact that, when the serum of a syphilitic is incubated with a Wassermann "antigen" flocculation appears in the mixture. Of these the Sachs-Georgi was the original, and among others are those of Meinicke and Vernes, and the Sigma Reaction of Dreyer and Ward.

Within the past few years, however, the Kahn test has established itself as the most reliable and most widely used of the flocculation reactions. For it only three reagents are required :—

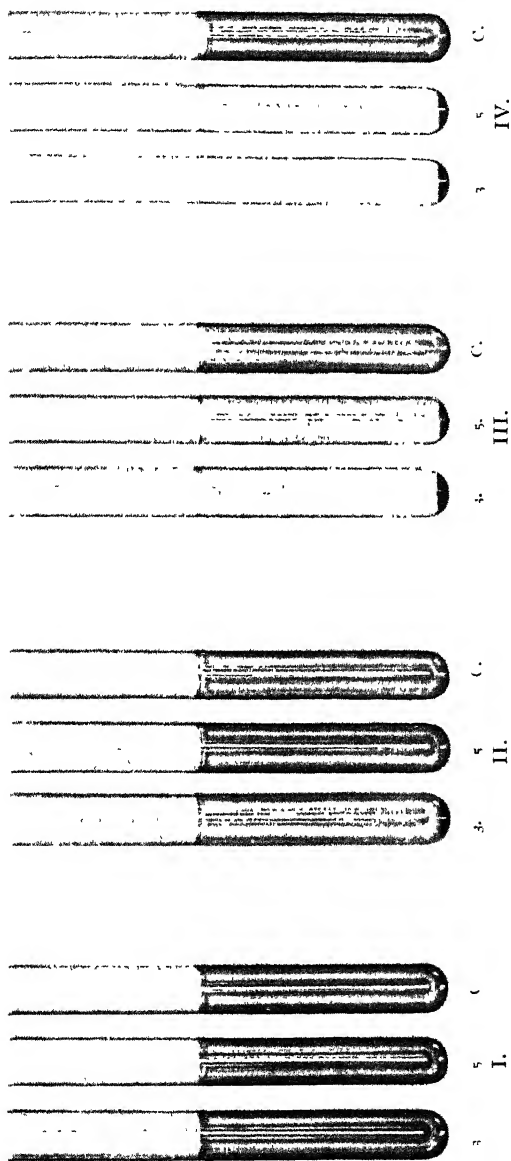
1. *Patient's serum*.—This is obtained in the same way as for the Wassermann reaction, and is heated at  $56^{\circ}\text{C}$ . for half an hour.

2. *Saline*.—0.85 per cent. sodium chloride solution.

3. *Antigen*.—This consists of an alcoholic extract of beef heart (previously treated with ether) to which 0.6 per cent. of cholesterol is added. The details of the preparation cannot be described here, and those interested are referred to "The Kahn Test" by R. L. Kahn. The prepared antigen can be purchased through the usual channels. It must be kept in bottles with tightly fitting stoppers or rubber bungs covered with tinfoil. For use the antigen is diluted with saline, the degree of dilution needed for any particular batch of antigen being described as its titre. Usually 1.0 c.c. of antigen requires from 1.0 to 1.3 c.c.s. of saline.

To carry out the test 1.0 c.c. of antigen is measured into a 5.5 cms.  $\times$  1.5 cm. flat-bottom vial, and the amount of saline indicated by the antigen's titre (say 1.2 c.c.) into another similar vial. The saline is poured into the vial containing antigen, and the mixture rapidly poured back. This double operation is repeated six times rapidly, in no case waiting to drain a vial completely. The antigen so diluted is allowed to stand for ten minutes before using: it must not be used more than thirty minutes after dilution. It is then well shaken, with the thumb on the end of the vial, and measured into the tubes used for the test. These tubes measure 7.5 cms.  $\times$  1.0 cm. Three tubes, arranged one behind the other in a rack, are used for each serum tested. 0.05 c.c. of dilute antigen is measured into the front tube, 0.025 c.c. into the middle, and 0.0125 c.c. into the back, finely graduated pipettes being used and the amounts being delivered to the bottom of the tubes. Without delay (*i.e.* as soon as the antigen has been measured into the ten sets of three tubes which the rack holds) 0.15 c.c. of each serum is measured into each of the three tubes used for testing that serum, and the rack is vigorously shaken by hand for ten seconds. Where the air temperature of the laboratory is below 21° C. (as it usually is in Europe in the winter), it is now advisable to place the racks in a water bath at 37° C. for ten minutes. With

# PLATE II.



## The Wassermann Test.

I. = - ; II. = + ; III. = ++ ; IV. = +++.

(3 - tube with 3 M.H.D. of complement ; 5 - tube with 5 M.H.D. of complement ; C. = control tube.)



higher air temperature this is unnecessary. The racks are then vigorously shaken (by hand or in a machine) for three minutes at from 275 to 285 oscillations per minute. 1.0 c.c. of saline is added to each front tube, and 0.5 c.c. to each middle and back tube, and the racks shaken by hand sufficiently to mix the contents of the tubes. The results may then be read. In strongly positive reactions, flocculation is easily visible to the naked eye, even without removing the tube from its rack. In negative reactions the fluid is uniformly opalescent. For reading results we prefer holding the tube almost horizontally about  $1\frac{1}{2}$  inches above a concave mirror and looking at the image in the mirror. This gives good illumination and sufficient magnification. For each serum tested the reactions occurring in the three tubes are recorded on the following scale:—

- ++++ = particles easily visible to naked eye.
- +++ = smaller particles but still visible to naked eye.
- ++ = fine particles on the border-line of visibility without magnification.
- + = particles visible only with magnification.
- ± = doubtful.
- = uniform opalescence.

The strongest reactions may occur in either the front or back tube of the three. To obtain the final result for the serum the number of + signs recorded for the three tubes is added and divided by 3. In case this gives a fraction, if the fraction is  $\frac{1}{3}$ , it is disregarded, if  $\frac{2}{3}$  an extra + is added to the whole number.

Front.	Middle.	Back.	Final result.
+++,	+++,	++++	= +++
+,	++,	++	= ++
+++,	+,	—	= +
+,	+,	++	= +
—,	—,	+	= —

++ or over is regarded as positive, + or ± as doubtful, and — as negative. The clinical interpretation of results is considered in chapter XL.

## CHAPTER VIII

### THE OBTAINING OF MATERIAL FROM THE PATIENT FOR BACTERIOLOGICAL EXAMINATION

THE ultimate aim of much of our practical bacteriological work is to be able to identify with absolute certainty the organism responsible for some pathological condition. The universality of bacteria must be borne in mind in collecting material for bacteriological examination. We wish to obtain the pathogen responsible for the disease ; we must be on our guard to exclude, so far as possible, bacteria which are saprophytic or merely parasitic. Obviously, if we examine the fæces for pathogenic organisms we cannot obtain a specimen without a large number of the normal intestinal types ; but if we collect pus from an abscess we should avoid the bacteria occurring on the skin, or if we propose to examine urine we should endeavour to obtain that fluid as it exists in the bladder, avoiding the organisms found in the terminal portion of the urethra.

For the collection of material for bacteriological examination we should provide ourselves with a supply of sterile apparatus of which the most useful are plugged test tubes, cotton swabs on wires, capillary pipettes, and corked bottles. The methods of obtaining material from certain localities will now be mentioned.

**Throat Swab.**—The patient's head is thrown slightly backwards, the tongue depressed with a spatula, and the patient asked to say "ah—ah—ah—ah !" In this way the soft palate is elevated, and the swab held by the wire is withdrawn from its tube and introduced into the mouth. It is rubbed firmly on the suspected part, whether the tonsil, supra-

tonsillar fossa, uvula or elsewhere, withdrawn, avoiding the tongue and lips, and restored to its tube. If *C. diphtheriæ* is to be searched for, the swab should be used to inoculate a tube of coagulated serum and also one of a tellurite medium, both of which are then incubated. If we desire information as to the general bacteriology of the part, a blood-agar plate is inoculated from the swab and subsequently spread. In all cases some direct films should also be made from the swab, and one of these may be stained by Gram's method. If this precaution is not taken, the occurrence of the organisms associated with Vincent's angina may be overlooked. Swabs from the naso-pharynx may be similarly obtained, but the swab should have its terminal centimetre bent almost at a right angle; this is hooked under the soft palate, which should not be touched, and rubbed over the posterior naso-pharyngeal wall. A West's swab is sometimes used: this is made of thin, flexible wire and is protected by a glass tube the terminal portion of which is bent almost at a right angle. The swab is held within the tube until the soft palate is passed, when it is pushed out and rubbed over the wall of the naso-pharynx. It is then retracted within the protecting tube and the whole withdrawn. The obtaining of a swab from the larynx in cases of suspected laryngeal diphtheria should be left to a laryngologist.

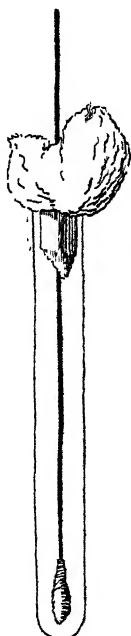


FIG. 29.—  
STERILE SWAB.

**Pus from an Abscess and other Fluids.**—The skin should be disinfected as thoroughly as possible and the disinfectant removed with alcohol. When the surgeon has made the opening the pus may be allowed to flow into a sterile tube if large in amount or, if not much is present, some may be collected on a swab. Fluid such as that found in a vesicle may be collected in a sterile capillary pipette. The end of the pipette is sealed in order to allow the fluid to be carried to the laboratory. In all cases where a swab is used, unless it can be



examined with very little delay, it should, before the pus is collected, be moistened with sterile saline to prevent drying. For an internal abscess a swab with a long wire handle is most useful for obtaining a sample of the pus. However obtained, films of the pus should be stained and cultures made on blood-agar or other medium suggested by the microscopic examination.

**Sputum.**—The mouth should be rinsed out with warm boiled water (not containing any antiseptic) and the throat gargled with the same. By deep coughing, material should be brought up from the deeper parts of the respiratory tract and expectorated into a sterile wide-mouthed bottle with a rubber bung. For examination, the sputum must be emptied into a sterile Petri dish and, if cultures are required, a selected mass of purulent material should be taken with the platinum loop and rinsed in three or four changes of sterile saline to free it from contaminating organisms on its surface. It may then be plated on blood-agar. A similar mass of purulent material should be used for making films, whether for the detection of tubercle bacilli or other bacteria.

**Fluid from Serous Cavities** (Pleura, peritoneum, etc.).—This should be obtained with a syringe and needle, preferably an all-glass syringe autoclaved or dry sterilized ready assembled (see Fig. 30). After the skin has been treated with iodine, the needle may be introduced into the cavity and the fluid aspirated. When the needle is withdrawn, the fluid should be expelled immediately into a sterile test tube. When such fluid is almost clear (not purulent) it frequently clots, and this may interfere with its examination. It is therefore advisable to divide it into two portions, one of which is received in a dry sterile tube and the other in a sterile tube containing 1 to 2 c.cs. of citrate solution. Direct examination of wet films is useful in determining the nature of the leucocytes present. If these are few, some of the fluid should be centrifuged at a high rate for a few minutes and films made from the deposit. Where Gram's method fails to reveal any bacteria, cultures should be made on blood-agar and a number of films examined for the presence of tubercle bacilli. Cerebro-spinal fluid is usually obtained with a special long needle 1 mm. in bore. This is introduced

into the canal through the fourth lumbar space, the patient's back being bent so as to increase the distance between the vertebræ, and the fluid emerging is collected in a sterile test tube. Since the first few drops frequently contain red blood corpuscles, it is well to collect the fluid in a series of two or three tubes, the latter of which will probably be free of these. The method of examining this fluid is similar to that used in the case of pleural fluid, but examinations should be made quickly if the meningococcus is suspected. In the case of tuberculous meningitis, the tubercle bacilli can almost invariably be found by prolonged search of films made from the centrifuged deposit or from the fine threads which form on standing. Where this fails, as in the case of pleural fluid where failure is common, and the cultures are sterile, a guinea-pig should be injected with the deposit. In this way tuberculosis may often be detected where microscopic examination has given negative results. The nature of the cells present is suggestive of the infective organism, since a predominance of lymphocytes in a clear fluid is usual in tuberculosis and of polymorphonuclear leucocytes in a turbid fluid in more acute infections. In very acute cases of tuberculous meningitis, however, polymorphonuclear leucocytes may predominate.

**Urine.**—It is always preferable to obtain specimens of urine by a sterile catheter, but, in the male, washing of the glans and meatus with soap and water, the rejecting of the first portion voided, and the collection of the latter part in a sterile flask, is quite satisfactory if the specimen is to be examined immediately. Catheterization is essential in the female. Direct examination of a drop of urine in a case of cystitis will usually reveal pus cells and bacteria and a culture made from a loopful will generally give many colonies. In some cases, however, the centrifuge may be necessary in order to obtain pus cells and bacteria in sufficient numbers for microscopic examination. Films made from urine should, after fixing, be well washed in water (which may be gently warmed on the slide by applying a small flame beneath). This removes much of the unorganized deposits. The films may then be stained as described. By doing this, clearer

and more satisfactory microscopic preparations are obtained than by staining the film directly after fixing. Where the culture from the uncentrifuged urine shows only one or two colonies these are to be regarded with doubt. Blood agar plates, especially for the gonococcus, and ordinary agar for the majority of other bacteria may be used. The centrifuged deposit should be used for films for the detection of tubercle bacilli. In tuberculosis of the urinary tract these are generally fairly numerous; but one must be guarded, since the smegma bacillus is commonly found about the external genitals, and this organism is also acid-fast. The best precautions are thorough preparation of the part, the use of a catheter and, in the Ziehl-Neelsen method, a full treatment with alcohol. In case of doubt, the only absolutely reliable method is the injection of a guinea-pig; this method has the further advantage over the direct microscopic examination that it gives a higher percentage of successes.

**Fæces.**—The stool should be passed into a clean vessel and a small portion removed with the metal spoon attached to the cork of the collecting outfit. If dysentery bacilli are sought, a portion of blood-stained mucus should be isolated, washed in several changes of sterile saline, and plated on MacConkey's or other suitable medium, using dried plates. For members of the enteric and food-poisoning groups, two tubes of tetrathionate broth should be inoculated, one heavily and the other lightly. Plates of Wilson and Blair's medium should be spread, one from each tube, without delay. The tubes should be incubated for twenty-four hours when a MacConkey plate should be spread from each. Wilson and Blair plates should be incubated for forty-eight hours, MacConkey plates for twenty-four hours. The rapid growth of *V. cholerae* on the surface of peptone water is used to secure enrichment of that organism as a preliminary to plating. Streptococci may usually be isolated from fæces by culturing a small portion in glucose hydrocele broth. The deposit after twenty-four hours' incubation should be plated on blood agar. Tubercle bacilli are not commonly found in films made directly from fæces. Concentration methods (see Chapter XXXVII.) give

better results, but the only reliable method is to treat with 5 per cent. caustic potash or 3·4 per cent. sulphuric acid and to inject the deposit obtained by centrifuging into a guinea-pig.

In many cases, particularly in the examination of suspected carriers of the enteric bacilli, the previous administration of a purgative followed by salts will give a stool more likely to contain the organism than one obtained without purgation. The result obtained by a simple soap enema is also frequently useful for culturing the enteric bacilli from a patient or carrier.

**Genitals.**—The commonest examinations made are for the organisms causing venereal disease, particularly the gonococcus and the *Tr. pallidum*. The gonococcus, in the male, is present in the urethral discharge. The glans and meatus are well washed with sterile water or saline, and some pus is expressed by the patient massaging the urethra from behind forwards; this is collected on a platinum loop and, if a culture is desired, is immediately spread on a freshly made moist blood agar plate which is at once incubated, preferably in an atmosphere containing 10 per cent. of carbon dioxide. Films are made and stained by Gram's method. If no pus is obtained in this way, it may be provided by massaging the prostate from the rectum. In the female, material should be taken on a swab or platinum loop from the urethra, the cervix, or the mouth of Bartholin's gland, not from the vagina.

If a suspected chancre is present, it should be well cleansed with saline, squeezed vigorously and rubbed with sterile gauze. Some oozing of blood may occur, and this should be absorbed. Soon the blood ceases to flow and a drop of clear fluid appears. This is collected with a fine capillary pipette and used for examination by dark ground illumination or for preparing films for staining. Rubber gloves should be worn to prevent accidental infection.

**Blood Culture.**—The syringe, an all-glass one with a really sharp needle, should be assembled, placed in a large glass test tube and sterilized in the autoclave or dry oven. Only in emergencies should boiling be relied upon. 100 c.cs. of broth (either ordinary broth or, if the enteric group be suspected, broth containing bile or 0·5 per cent. sodium

taurocholate, which inhibits the bactericidal action of the patient's blood) should be ready in a special blood culture bottle which is prepared from a 6-ounce screw-cap medicine bottle. The cork washer is removed and a rubber washer is cut to the same size, and a  $\frac{1}{4}$ -inch hole punched in the metal cap. The bottle is about half filled with broth, the washer adjusted, and the cap screwed home, the rubber washer now being visible through the hole in the metal. A stout linen thread is tied around the neck of the bottle and brought over the top, and a cellulose cap (such as the viskap, manufactured by the Viscose Development Co., Bromley, Kent) is placed over the metal cap. The whole is autoclaved, and it will be found, after drying, that the viskap has contracted, forming a firm covering over the metal cap and

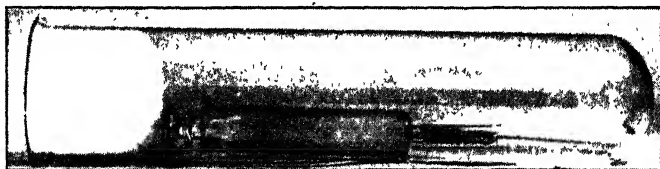


FIG. 30.—ALL-GLASS SYRINGE STERILIZED IN LARGE TEST TUBE, READY FOR PERFORMING A BLOOD CULTURE.

neck of the bottle, which prevents bacterial contamination of the exposed portion of the rubber washer. It is of importance to have as large a volume of broth as possible in the bottle so that any antibacterial substances present in the patient's blood, which might interfere with the growth of the bacteria outside the body, may be diluted to such an extent as to render them inactive. The skin in the region of the elbow is treated with tincture of iodine and a tourniquet applied above this. The patient's arm is extended and, if no veins are prominent, it is allowed to hang down for a few moments. The syringe is withdrawn from the tube and the stilette removed from the needle. The skin is stretched tightly to one side of the selected vein and the needle entered first through the skin, passed on a short distance in the subcutaneous tissue, and then into the vein. The left hand which stretched the skin is now

transferred to the syringe and the right slowly draws out the plunger, filling the syringe with blood. When full, the tourniquet is removed and the needle sharply withdrawn. If the puncture has been properly made there will be no bleeding, owing to the valvular nature of the opening; but if a few drops of blood ooze out, tight pressure on the spot with a piece of cotton wool will quickly cause the flow to cease. The thread projecting from under the viskap of the bottle is pulled, so cutting the cap and usually causing it to fall off.



FIG. 31.—BLOOD CULTURE BOTTLE.

This exposes the rubber (which is sterile) in the centre of the metal cap. The rubber is pierced with the needle and 5 to 10 c.cs. of blood injected into the bottle from the syringe. When the needle is withdrawn the minute hole in the rubber seals itself. The bottle is shaken to diffuse the blood through the medium and incubated. After incubation, the broth is examined microscopically and subcultures made on blood-agar or other medium. Blood cultures are useful in septicæmia, whether due to streptococci, staphylococci or other bacteria; in the diagnosis of enteric fevers, undulant fever, and in other

conditions where it is believed that bacteria may be present in the blood.

**Collecting Serum for Wassermann Tests, Agglutination, etc.**—An all-glass, or Record syringe, is sterilized by boiling either in water or in saline. If in water, it is essential to work the plunger gently up and down while the syringe is still hot, in order to expel all water which would cause hæmolysis. 10 c.cs. of blood are collected, as described in the technique of blood culture, and are expelled into a dry sterile tube. All bubbling and frothing must be avoided and, if some air is present with the blood in the syringe, it must not be expelled with the blood into the tube. The tube should be allowed to stand without agitation until clotting has taken place, when the clot may be gently loosened from the sides of the tube with a sterile wire or fine glass rod. It will then contract and, if the tube is left at room temperature until the next day, the clot will be found to have shrunk and to be covered and surrounded by clear serum free from red blood cells. This may be removed to a fresh tube by means of a sterile capillary pipette. The serum should be of a light yellow colour; if reddish, hæmolysis has occurred, either owing to the accidental addition of water, alcohol or other substance, to rough handling, or to bacterial contamination. If the serum is required quickly, the tube should be placed for two or three hours in the incubator or water-bath at 37° C. At that temperature coagulation occurs rapidly and the clot quickly expresses the serum. At the end of that time the serum which surrounds the clot and which contains red blood corpuscles is pipetted off and the red cells deposited by centrifuging, when the clear supernatant serum can be removed. In taking blood for any serological test it is advisable to avoid the few hours following a meal, as at this time the serum may be heavily loaded with fat which may interfere with the test.

The Behring venule affords a very convenient method of collecting blood for serological tests. It consists of an evacuated receiving tube with a rubber stopper, through which passes a fine glass tube to the end of which a needle is fused. The fine tube is so arranged that its inner end is in contact with

the rubber of the stopper, so forming a valve which prevents entry of air into the receiving tube unless the projecting fine glass tube is bent in relation to the rubber stopper. The needle is protected with a covering glass tube, and the whole

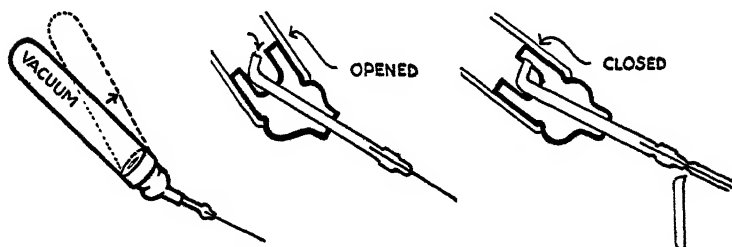


FIG. 32.—BEHRING VENULE.

is sterile. For use, the tube protecting the needle is filed and removed, and the needle introduced into a vein. The connecting tube is then bent on the stopper and blood enters and fills the receiving tube. For transmission the needle may be removed and the outfit sent through the post.

**Tissues and Organs obtained at Operation or Post-mortem Examination.**—Material such as this is very liable to have its exterior contaminated, and so it is necessary to obtain material for culture from the interior. The best method is to sear an area with the cautery or a soldering-iron. This can be cut through with a sterile knife and material taken from the interior, or the seared part may be punctured with a sharp sterile capillary pipette and a few drops of fluid aspirated. In this way material for culture may be obtained from solid organs such as the spleen, or from the heart either of man or animal at post-mortem examination.



## CHAPTER IX

### THE IDENTIFICATION OF PATHOGENIC BACTERIA

It is impossible in bacteriology to devise a definite scheme of tests, the careful application of which will inevitably lead us to the correct answer to the question "What is this organism?" Nevertheless we should endeavour to work in as logical a manner as possible. The first step is a careful examination of the material in which the organism occurs, and in this connection the bacteriologist must, early in his career, learn to use some, if not all, his senses. The employment of the eyes is essential, of the sense of touch through the intermediary of the platinum wire, the slide, or other implement, is useful, and of the nose, is by no means to be despised: taste and hearing to the bacteriologist are of little importance. In connection with the use of vision do not let us, because we deal with very minute objects, despise the naked eye, the hand lens or the low power of the microscope. The material should be thoroughly examined, for frequently from the colour, consistency or smell of pus, or from the turbidity of urine or cerebro-spinal fluid, we may derive much valuable information. Direct examination of a specimen of pus with the naked eye and with the hand lens may save us from overlooking the presence of the granules of actinomyces, which we may ignore if we merely make films and cultures from unselected parts. In the case of sputum the examination of carefully selected portions is very much more valuable than the preparations of films or cultures from random samples. The colour of pus may suggest such bacteria as the *Ps. pyocyanea*, and its smell may direct our attention to certain anaerobic bacteria.

After these preliminary observations we may proceed to the use of the microscope. An unstained wet preparation may

frequently be of assistance, particularly in the case of such fluids as urine, since by its help we may ascertain the presence or absence of pus cells or other leucocytes and of red blood corpuscles. In many cases we may see in this preparation the bacteria themselves; we may be able to determine their morphology, whether bacilli or cocci and, if the former, whether motile, if the latter, whether streptococci or staphylococci or of uncertain grouping. In some cases, as in syphilis, it is possible to make a definite diagnosis of the disease from unstained films examined by dark ground illumination. The student is liable to think much of this waste of time and desire to arrive straight away at the next step—the staining of films.

In the examination of some material (fæces, for example, for the presence of bacilli of the enteric or dysentery groups) films do not yield any information of value; but in the great majority of cases films should be prepared, suitably stained and examined before cultures are made, as our cultural methods depend to a considerable extent on the variety of bacteria present. Simple stains are capable of furnishing a certain amount of information as to the morphology of the bacteria present; but Gram's method should also be used as a routine, as by its help we are able to classify the majority of bacteria into two groups—the Gram positive and the Gram negative. The pathogenic cocci, except the gonococcus, meningococcus and *N. catarrhalis*, are gram positive. Of the bacilli *C. diphtheriæ*, *B. anthracis*, tubercle bacillus, *Myco. lepræ* and the anaerobic bacilli are Gram positive: most other pathogenic bacilli are Gram negative. The *V. cholerae* and all spirochaetes capable of being stained with simple stains are Gram negative.

Certain bacteria do not stain well, if at all, by either the stain or the counter-stain used in Gram's method. In this class we may place, as of great estimportance, the *Myco. tuberculosis*, many of the other acid-fast bacilli and the majority of the spirochaetes. In examining pus, for example, when simple stains and Gram's stain do not reveal any bacteria it is well to stain a film by the Ziehl-Neelsen method. The presence of spirochaetes is frequently first suspected on clinical grounds,

and the special methods, such as Fontana's, are not usually employed, except when we are expressly asked to ascertain the presence or absence of these organisms. Protozoa in the blood are usually discovered by direct microscopic examination of films, stained with Leishman's stain. In some cases special staining methods, such as that to demonstrate capsules, may be employed on films made from the material under examination.

We may now summarize the information which we can obtain by the microscopic examination of bacteria in preparations made either from the original material or from cultures. The size, shape and arrangement of the bacteria are first observed. The relatively large *B. anthracis* can hardly be confused with the much smaller *Bact. coli* or the minute *H. influenzae*. The short oval of *Past. pestis* contrasts with the elongated rectangle of *B. anthracis*. *Bact. coli* is usually found as single elements, *B. anthracis* in chains and *C. diphtheriae* in characteristic clusters. Unstained preparations help to differentiate *Bact. typhosum* from *Bact. flexneri* on account of the motility of the former. The presence of a capsule differentiates *Bact. pneumoniae* from *Bact. coli*. The presence or absence of spores and, if present, their shape, size and position are of great assistance in identification. The large spherical and terminal spore of *Cl. tetani*, for example, is very characteristic. The importance of Gram's stain has already been mentioned, but other differential staining methods, especially that of Ziehl and Neelsen, by which, in the majority of cases, the tubercle bacillus is identified, are also of real service. Finally, the regularity or otherwise of staining of the bacterial cytoplasm—whether beaded, as in *C. diphtheriae*, or chiefly polar, as in *Past. pestis*, is, in certain cases, of considerable help.

Frequently these preliminary examinations will suffice for the identification of the organism for purposes of diagnosis. In general, however, cultures are essential either for the complete identification of the organism, for the preparation of a vaccine, or for other purposes. The success of a culture depends to a considerable extent on the freshness of the material when cultured and the suitability of the cultural methods. As a

routine medium for the cultivation of the organisms causing disease in man, nutrient agar may be used. In practically every case, however, it is more satisfactory to use blood agar. Where the information furnished either from the clinical history of the case, the source of the material, or the preliminary examination, points to a certain organism or group of organisms, special methods may be employed, such as MacConkey's or Wilson and Blair's media for intestinal bacteria, solidified serum for diphtheria bacilli, minced meat broth for anaerobic bacteria, and glucose hydrocele broth for streptococci.

Incubation for 24 to 72 hours is, in general, sufficient. The plates or other cultures should be examined and, if the former have been properly prepared, well-isolated colonies should be seen. The examination of colonies with the naked eye, hand lens or low power of the microscope as well as the consistency of the colony, as judged by touching with a platinum wire, are of the highest importance in identifying bacteria. Such points as the colour, size, shape, outline (whether even, crenated or irregular), surface (whether smooth, shiny or rough), whether transparent or opaque, whether elevated, flat or depressed in the centre and whether dry or moist, should all be carefully noted. It should be observed whether the colony is sticky, tough, or lumpy and difficult to break up, and whether it is easily removed or adherent to the medium.

If blood agar is employed any alteration produced in the medium is of great importance. Some bacteria cause hæmolysis of the blood cells distributed through the agar owing to the diffusion of a hæmolysin. The result is that the plate shows a zone of clearing around the colony, but this zone is still of a blood-red colour. Such a change is shown in cultures of certain bacteria. In what is commonly called hæmolysis, however, something more than mere laking of the cells has occurred, as the zone not only becomes clear, but also loses almost all its red colour. Some of the hæmoglobin has been absorbed into the colony, some has diffused into the surrounding medium, but a considerable part has been altered into a colourless compound as a result of hæmodigestion. On blood

agar some bacteria (*e.g.* pneumococci) produce a green coloration. This is due to some oxidation product of hæmatin which results from the action of  $H_2O_2$ , produced by the bacteria, on a hæmoglobin derivative.

While the appearance of the colony is an important characteristic of an organism, wide variations from the normal are sometimes observed. In the case of the intestinal bacilli the normal type of colony has an even outline and a smooth shining surface, but variants, in which the outline is crenated and the surface irregular and rough, are sometimes obtained by plating material from the body or from cultures in fluid media of unsuitable composition or containing immune

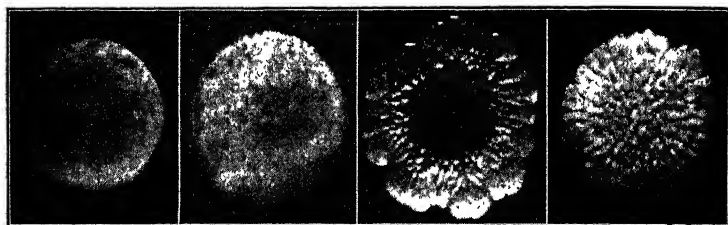
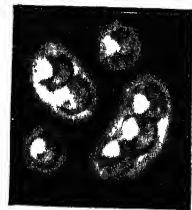


FIG. 33.—VARIANT COLONIES OF STAPHYLOCOCCUS PYOGENES.

serum. Examples of "rough" colonies of staphylococci are seen in Fig. 33. It has been observed that while smooth, normal colonies form an even stable suspension in saline, the rough types form suspensions which are granular and spontaneously agglutinating, although stable suspensions may usually be obtained by decreasing the strength of salt in the solution. We shall have occasion later to refer to antigenic differences and to alterations in virulence which occur when variant colonies are produced.

When the colonies on the plate have been carefully examined, films of each variety should be prepared and stained by Gram's method. After this, it may be possible to state definitely what the organism is; but if several types of colonies are present it is usual to obtain each variety of organism in pure culture before further investigations are carried out. In some instances it is useful to con-

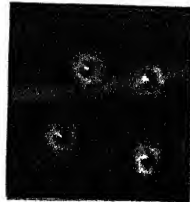
# PLATE III.



1.



2.



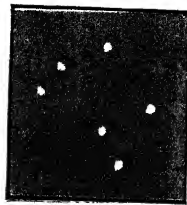
3.



4.



5.



6.



7.



8.

## Colonies of Bacteria.

1. *Staphylococcus pyogenes* on blood agar.
2. *Streptococcus pyogenes* on blood agar.
3. *Streptococcus viridans* on blood agar.
4. *B. typhosus* on Wilson and Blair's medium.
5. *B. coli* on MacConkey's medium.
6. *B. typhosus* on MacConkey's medium.
7. *B. diptheriae* (Gravis) on McLeod's medium.
8. *B. diptheriae* (Mitis) on McLeod's medium.



trast the morphology of the organism when grown on different media (streptococci usually produce longer chains in fluids than on solids). The production of spores should be tested both in stained films and by finding the thermal death-point of the organisms. The temperature of growth (maximum, optimum, or more usually minimum) may be of use. The gonococcus, for example, will only grow at approximately the temperature of the body, while the *N. catarrhalis* will also grow at 20° to 22° C. Another point is the variety of medium on or in which good growth is obtained, such as whether the organism will grow on plain agar or demands an enriched medium, and whether it grows only or better under aerobic or anaerobic conditions. The naked eye appearance of cultures of certain organisms on special media is useful. The pellicle of *V. cholerae*, the uniform turbidity of *Bact. typhosum* and the deposit of streptococci in broth culture are suggestive indications of the organism in the culture. The microscopic examination of a broth culture is the most reliable method of determining whether or not a bacterium is motile.

Gelatin cultures are useful in showing us whether the bacteria we are investigating liquefy that substance or not. The *V. cholerae*, *B. anthracis*, staphylococci, and *Proteus vulgaris* cause liquefaction, the streptococci and the coli, enteric and dysentery bacilli do not.

The products of growth are also utilized in the identification of bacteria. Among these may be mentioned the production



FIG. 34.—ON LEFT, UNIFORM TURBIDITY OF *BACT. TYPHOSUM* IN BROTH, ON RIGHT DEPOSIT OF STREPTOCOCCI IN BROTH.



of indol in broth or peptone water. This may be tested for by the Nitroso-indol method: to the broth culture are added a few drops of a 0.1 per cent. solution of potassium nitrite and a drop of sulphuric acid, when a rose colour indicates the presence of indol. A more sensitive test is Ehrlich's Rosindol Reaction, in which the reagent used is—

Paradimethylamidobenzaldehyde	1.0 gm.
Absolute alcohol	95.0 c.cs.
Concentrated hydrochloric acid	20.0 c.cs.

This reagent is carefully added to the broth with a pipette so that it floats on the surface, and the tube is gently agitated to secure a slight mixing between the fluids. If indol is present a red ring develops and the colour diffuses through the supernatant solution. The production of indol is particularly useful in the identification of certain members of the intestinal group of bacilli; it distinguishes *Bact. flexneri* from *Bact. typhosum* which in many other respects behaves similarly. If, in addition to indol, nitrites are also produced, the nitroso-indol reaction is given on the addition of sulphuric acid alone. This is known as the "Cholera Red Reaction," and distinguishes the *V. cholerae* from many other vibrios which it resembles.

The Voges and Proskauer reaction is useful in distinguishing certain members of the group of intestinal bacilli. The test is carried out by growing the organism for one or two days in the buffered glucose broth described on p. 48. To the culture is added a knife point of creatin (as recommended by O'Meara) and an equal volume of 40 per cent. sodium hydrate solution. The whole is vigorously shaken and, if the test be positive, a red colour develops in a few minutes. A positive reaction is due to the production of acetyl-methyl-carbinol. A satisfactory alternative method of demonstrating the production of acetyl-methyl-carbinol is that of Barritt. To 1.0 c.c. of a culture in buffered glucose broth are added 0.6 c.c. of a 5 per cent. solution of  $\alpha$ -naphthol in alcohol and 0.2 c.c. of a 40 per cent. solution of potassium hydrate. The tube is vigorously shaken and, if the result

is positive, the fluid becomes pink in a few minutes, the colour deepening to crimson or magenta within one hour.

The fermentative activity of an organism is, in the case of the members of some groups, of outstanding utility for identification. To suitable medium (peptone water most usually) 0.5 to 1.0 per cent. of various carbohydrates are added and the medium then used for the cultivation of the organism. It may either fail to act on the carbohydrate or it may ferment it, producing either acid alone or gas as well as acid. The production of acid may be determined by titration or, as is more usually done, by the addition to the medium of an indicator which changes colour when acid is produced. If gas is evolved, it is collected in a small test tube inverted in the medium (*see* Plate IV). The gas is generally a mixture of hydrogen and carbon dioxide and the proportion of the two may be of some importance. Gas production may also be judged by the splitting caused in solid media containing fermentable carbohydrates, and when the amount of gas is small this method is more delicate than that in which fluid media are used (*see* Fig. 11). The determination of the fermentative activities of the organism is most useful in the case of the Gram negative intestinal bacilli in which morphology assists very little in identification.

The methyl-red test depends on the final reaction of the medium after glucose fermentation. Buffered glucose broth (p. 48) is inoculated with the organism and incubated for four days. A few drops of methyl-red solution are added and the resulting colour noted. If red (showing persisting acidity), the test is positive; if yellow (indicating that the primary acidity has been replaced by alkalinity), it is negative. This test is chiefly used for distinguishing coliform bacilli isolated from water.

As a result of the various procedures described we are, at this stage of the investigation, either quite satisfied as to the identity of the organism or have narrowed the choice down to two or three; the next appeal is frequently to serology. When an animal, such as a rabbit, is inoculated with an organism, antibodies are produced to that organism and are found in

the animal's serum. The antibodies most useful in determinative bacteriology are agglutinins, and without difficulty we can furnish ourselves with a supply of agglutinating sera prepared against various standard organisms. If an unknown organism, suspected on other grounds of being the *Bact. typhosum*, is agglutinated by an anti-typhoid serum, we can, in practically every case, decide definitely that the bacillus is the *Bact. typhosum*. The macroscopic method should be used. If the titre of the serum is, say,  $\frac{1}{3000}$  against its own organism, it should agglutinate our organism, if the two are identical, to at least  $\frac{1}{1000}$ . It is essential to carry the dilution of the serum in the test to its titre, as agglutination resulting with strong serum is much less reliable. In a few cases simple agglutination may be insufficient, owing to the presence of group agglutinins in the serum, and we then must fall back on the "absorption of agglutinin" reaction, the technique of which is described elsewhere. Sera may be used to establish the identity of an organism by complement fixation tests and by their bactericidal or bacteriolytic actions, but these are neither so simple nor so widely utilized as are the agglutinating properties of antisera.

The final test to be applied for the identification of an organism is frequently that of animal inoculation. Many bacteria which are pathogenic for man are also pathogenic for animals, and the results of infection with certain organisms, as seen at a post-mortem examination, may be sufficiently characteristic to make the presumptive identification certain. This is true, for example, of the tubercle bacillus, anthrax bacillus, pneumococcus and others. Those bacteria which produce a powerful exotoxin may be identified by finding that their cultures, freed from the bacteria themselves, produce a characteristically fatal result in one animal but not in another which has been injected simultaneously with antitoxic serum. This is the method used to prove that an organism resembling the *C. diphtheriæ* in morphology and cultural characteristics is actually a virulent *C. diphtheriæ*.

It is not necessary to employ all the methods described in every case. In the case of some bacteria it may be possible to

name the unknown organism definitely in a day or two ; in the case of others this may occupy a matter of weeks or even months. It is only by a fairly complete knowledge of the characteristics of the various types of bacteria, of the many cultural methods and of the niceties of technique, that the bacteriologist is able to select the methods of investigation necessary in each case and to avoid those not likely to yield useful information.

Under what conditions are we able to state positively that a given organism is the cause of a certain disease ? The most strict criteria are those known as Koch's Postulates.

1. The organism must be present in the tissues or fluids of the affected animal.
2. It must be isolated and cultivated outside the body for several generations.
3. The cultivated organism, on inoculation into a suitable animal, should reproduce the disease.
4. It should be again isolated from the artificially infected animal.

It is not possible in every case to fulfil all these requirements, but they represent the ideal to be aimed at. Since the postulates were formulated, immunology has given us some useful methods which help us to connect an organism with a disease, and every bacteriologist is satisfied that the causative organisms of several diseases are known although all the postulates have not been satisfied.

## CHAPTER X

### BACTERIOLOGY OF WATER, MILK AND SHELL-FISH

IN counting the number of bacteria in a fluid one may ascertain either the total number or the number of living bacteria in a given volume. Counts of the living organisms are of much greater value in connection with the examination of water and milk.

There are two chief methods used for "living" counts. In the first, various dilutions of the fluid in sterile water are added to tubes of solid medium after these have been melted and cooled, and from these plates are poured. After incubation, the number of colonies is counted and hence the total number of living bacteria found, it being assumed that each colony arose from a single organism, an assumption not entirely justifiable. In the second, decreasing amounts of the fluid are added to tubes of liquid medium and these are incubated. The earlier tubes will show evidence of growth; but, as the quantity of fluid decreases, a tube is reached in which no bacteria were added to the medium, and this, therefore, remains sterile. By presuming that the last tube showing growth received one organism we can calculate the total number present. Certain objections exist to both these methods, the chief of which is that no single medium and no single temperature of cultivation is suitable for all varieties of bacteria, and therefore the counts are too low. The first method would appear the more accurate; but by making a number of plates, each containing the same amount of the fluid, considerable differences in the number of colonies are found. In the second method only an approximation is attempted and the correct method of noting the results would be that bacteria were present in, say,  $\frac{1}{100}$  c.c. but not in  $\frac{1}{1000}$  c.c. This is commonly reported as "100 bacteria

present per c.c.," although the same result might be obtained if 500 were present. Irregularities exist owing to the impossibility of getting an absolutely even suspension and also, apart from this, they are inherent in the method. Suppose a fluid containing 1,000 bacteria per cubic centimetre is tested in this way, the quantity added to each tube being one-tenth of that added to the previous one; the tubes receiving 1.0, 0.1, 0.01 and 0.001 c.c. should be positive and those with 0.0001 c.c. and less negative. In practice, we might find that in some tubes receiving 0.001 c.c. the result would be positive and in others negative, and also we would occasionally find that in later tubes a few positives would occur because, although each one-thousandth of a cubic centimetre should hold only one organism, this bacterium occupies only a very small part of that volume and would be present in a particular ten-thousandth or one-millionth part of a cubic centimetre. It is advisable, in using this method, to duplicate each tube, so decreasing to some extent the errors of sampling. An alternative and better method is to inoculate a larger number of tubes with various quantities of the fluid as, by this method, the probable number of bacteria per 100 c.cs. may be ascertained. In making total counts solid medium is generally used, and in determining the numbers of special types of bacteria (indicator organisms) the fluid method is employed.

**Water.**—Water is usually examined in order to determine whether it has been subjected to faecal contamination which, while not dangerous in itself, renders the water liable to become the disseminating agent of various pathogenic bacteria, such as those of the enteric fevers and cholera. The water must be collected in a sterile bottle under conditions which will allow of the examination of a fair and representative sample. It should be examined as fresh as possible. Immediately before being examined the bottle should be vigorously shaken for half a minute.

Two sterile test tubes should be placed in a rack and 9.0 c.cs. of sterile water measured into the second. Some of the water to be examined is placed in the first and, with a sterile 1 c.c. pipette, 1.0 c.c. of this is added to the second and

the pipette returned to the first tube. With a fresh pipette the contents of the second are mixed. We thus have in the first tube the water and in the second water diluted  $\frac{1}{10}$ . Four tubes of agar are melted and cooled to  $48^{\circ}\text{C}$ ., 1.0 c.c. of water is measured into each of two plates and 1.0 c.c. of the  $\frac{1}{10}$  dilution into each of another two. With the usual precautions the agar from one tube is poured into each plate and the agar and fluid thoroughly mixed by a combination of rotation and rocking of the plate. One plate of each volume examined is incubated for two days at  $37^{\circ}\text{C}$ . and the others for three days at  $22^{\circ}\text{C}$ . The number of colonies in one plate of the two incubated at the same temperature is counted, the one selected showing, if possible, between 50 and 250 colonies. Where greater accuracy is required duplicate plates of each volume for each temperature should be made and the number of colonies averaged. The result should be reported as so many colonies developed per c.c. of water at  $22^{\circ}$  (or  $37^{\circ}$ ). The number should be stated to the nearest number with two significant digits. So, if 178 colonies are found in the plate containing 1.0 c.c. of the  $\frac{1}{10}$  dilution, the report would be "1,800 colonies per c.c."

In interpreting the counts the more important is that obtained at  $37^{\circ}\text{C}$ . The  $22^{\circ}$  count is, in itself, of little importance since many bacteria of soil and water grow at that temperature but not at  $37^{\circ}\text{C}$ ., but the ratio of the  $22^{\circ}$  to the  $37^{\circ}$  count is useful. It is impossible to lay down hard and fast standards since the number of colonies permissible varies with the class of water examined. In a deep well water, the  $37^{\circ}$  count should be low, probably below 5 per c.c., but much higher counts are to be expected in upland surface, river or shallow well waters. Any  $37^{\circ}$  count exceeding 50 should, however, be sufficient to condemn a water. Counts at  $22^{\circ}\text{C}$ . may be as high as several hundreds per c.c., even with perfectly good waters. In a first-class water, unless the  $37^{\circ}$  count is below 5, the ratio of  $22^{\circ}$  to  $37^{\circ}$  count should be high, 10 or more to 1, and may be as much as 30 to 1. In inferior waters the ratio is less than 10 to 1. With  $22^{\circ}$  and  $37^{\circ}$  counts of 300 and 15 respectively a water might be passed, while

counts of 50 and 15 would arouse suspicion. It should, however, be noted that the ratio is lower in summer than in winter and, in chlorinated water, the counts may approximate.

More important than the total colony count is the estimation of the number of characteristically faecal organisms present. These are known as "indicator" organisms, and the one most usually selected is *Bact. coli*. For the enumeration of this bacillus we use one bottle (with a tube inverted in it as in a Durham tube), five large and five small Durham tubes. The bottle should contain 50 c.cs. and the large tubes 10 c.cs. of double strength lactose bile broth. The smaller tubes should contain 10 c.cs. of ordinary lactose bile broth. To the bottle, 50 c.cs. of the water to be examined are added, to each of the large tubes, 10 c.cs. and to each of the small tubes, 1.0 c.c. All are incubated at 37° C. for 48 hours, and are then examined for the production of acid and gas, which is taken as presumptive evidence of the presence of *Bact. coli*.

From the number of tubes showing a positive presumptive *Bact. coli* result in the three volumes examined, it is possible to ascertain, by reference to McCrady's tables (given in the Ministry of Health's Report on "The Bacteriological Examination of Water Supplies," published by H.M. Stationery Office, London), the probable number of presumptive *Bact. coli* per 100 c.cs. of water.

*Bact. coli* is not the only lactose fermenting organism capable of giving a positive result in lactose bile broth and some of the other lactose fermenting bacilli, such as *Bact. aerogenes*, which may be found in faeces but more commonly gains access to water supplies from vegetation or the soil, are of much less sanitary significance than is *Bact. coli*. This organism is, however, absent from a pure water and its presence in unusually large numbers is, therefore, suspicious.

Three types of presumptive *Bact. coli* findings should be considered separately—those in which so few are present that the organism may be ignored, whether it be *Bact. coli* or *Bact. aerogenes*, and the water passed; those in which the count is so high that, even if the presumptive result is due to *Bact. aerogenes*, the water should be condemned; and an



intermediate type in which the judgment on the water will depend on whether the organism is, on the one hand, *Bact. coli* or a related, probably faecal, bacillus or, on the other, *Bact. aerogenes* or a related, probably non-faecal, organism. The report of the Ministry of Health fixed the levels at 2 and 10 respectively per 100 c.cs.—that is to say, a presumptive count of 2 or less should allow a water to pass, while a count of 10 or more should lead to its condemnation. As regards the lower limit we agree, but we are of opinion that 20 should be considered the limit beyond which a count should be considered as excessive, irrespective of the type of organism. Between 2 and 20 per 100 c.cs. is the border line region. If a presumptive *Bact. coli* count between these levels be obtained it is necessary to isolate the lactose fermenting bacillus and to establish its identity. This is done by plating a loopful from each positive tube or bottle on MacConkey's medium, incubating at 37° C for 24 hours and picking colonies for investigation.

Only bacilli which are Gram negative, non-sporing and non-gelatin liquefying need be further considered. The typical *Bact. coli* :—(1) produces indol ; (2) ferments dulcitol ; (3) does not ferment saccharose ; (4) gives a positive methyl red test ; (5) gives a negative Voges-Proskauer reaction ; (6) fails to grow in Koser's medium. If an organism having these characteristics or, at least, Nos. 4, 5 and 6, with any one of the other three, is present to the extent of 2 to 20 per 100 c.cs., it should be regarded as *Bact. coli* and the water should be condemned. If the organism responsible for the presumptive positive reaction differs in characteristics 4, 5 and 6, it may be regarded as *Bact. aerogenes*, its presence may be ignored and the water passed. There remain, however, a difficult group of intermediates in which the characteristics are neither those of a typical *Bact. coli* nor of a typical *Bact. aerogenes*. As regards these the bacteriologist must exercise caution, giving a guarded report which will tend towards condemnation when the presumptive figure is near the upper level (20) and when the characteristics incline towards those of *Bact. coli*, and towards approval when the presumptive figure is low and the characteristics approach those of *Bact. aerogenes*.

Since it is generally agreed that the presence in water of *Bact. aerogenes*, except in excessive numbers, is of little hygienic significance, any method which would permit *Bact. coli* but not *Bact. aerogenes* to grow in the tubes of lactose bile broth inoculated would greatly simplify the bacteriological examination of water. It is now fairly well established that *Bact. coli* can grow at 44° C., while *Bact. aerogenes* cannot. If we incubate the bottles and tubes of lactose bile broth in a water bath at 44° C., the development of acid and gas indicates, in the majority of cases, the presence, in the volume of water inoculated, of *Bact. coli* or of closely allied organisms.

Useful as the results of bacteriological examination of a water are, they do not render obsolete its chemical examination nor, above all, do they remove the necessity of a thorough topographical investigation of its source.

The examination of a single sample of water, unless where this has been collected correctly and the results are definitely bad, is of little value. A potentially dangerous water may occasionally give very good results. Bacteriological examination is most useful when tests of a given water supply are made repeatedly. Then a bacteriological standard for the particular water may be established and any unusual departure in an upward direction from this is an indication of the access of new contamination.

Members of the enteric group of bacilli have occasionally been isolated from a naturally infected water. Wilson and Blair's medium is most likely to be successful. One method is to mix equal volumes of the water and of the medium, melted, and cooled to 48° C., and to pour the mixture into plates. Another is to add to 100 c.cs. of the water, 0.5 c.c. of a 10 per cent. solution of alum. The precipitate which forms may either be spread on the surface of Wilson and Blair plates or be used to make deep plates by mixing it with the melted medium. Deep colonies of enteric bacilli in this medium are black. The cholera vibrio may be found by converting a considerable quantity of the water itself into the medium, by the addition of a concentrated solution of

peptone, the further stages being carried out in the manner described in the chapter on Cholera. Very rigorous tests must be applied before a vibrio isolated from water is definitely identified as the *V. cholerae*.

**Milk.**—By the bacteriological examination of milk much information may be obtained as to the cleanliness of its method of production and the care with which it has been kept. The most valuable tests are estimation of total bacteria growing in agar at 37° C. and of lactose-fermenting bacilli. Owing to the very great variations possible in the number of bacteria which may be present it may be necessary to employ higher dilutions than those given here. The following method will, however, give the necessary information for all but the most heavily contaminated milks.

Five sterile tubes are placed in a rack and 9 c.cs. of sterile water added to each, except the first, from a sterile 10 c.c. pipette. The milk is thoroughly shaken and some of it is placed in the first tube. With a sterile 1 c.c. pipette, 1.0 c.c. of milk is transferred from the first tube to the second and the pipette is returned to the first tube. With a fresh pipette the contents of the second tube are thoroughly mixed by drawing in and expelling from the pipette at least 12 times and also by bubbling air through the mixture. With this pipette 1.0 c.c. is transferred to the third tube and the pipette is returned to the second tube. This operation is repeated for each tube, a fresh pipette being used for each.

Tube No.	Dilution of milk.
1.	Milk.
2. 9.0 c.cs. saline + 1.0 c.c. milk	$\frac{1}{10}$
3. 9.0 c.cs. saline + 1.0 c.c. No. 2	$\frac{1}{100}$
4. 9.0 c.cs. saline + 1.0 c.c. No. 3	$\frac{1}{1000}$
5. 9.0 c.cs. saline + 1.0 c.c. No. 4	$\frac{1}{10000}$

1.0 c.c. amounts from tubes 2, 3, 4 and 5 are plated in agar, as was described in the case of water. Where the milk is believed to be of first class quality the plating from No. 5 tube, or even from Nos. 4 and 5, may be omitted. The plates are incubated at 37° C. for 48 hours and the number of colonies in

the one showing nearest to 200 is counted. This number, multiplied by the dilution used, represents the number of colonies developing at 37° C. from 1.0 c.c. of milk. It is usually stated as the number of bacteria per 1.0 c.c., but this is inaccurate as not all the bacteria in milk grow under the conditions used and also many of the colonies develop not from one bacterium but from a chain or clump.

Tubes of lactose bile salt broth are inoculated in duplicate with the following :—

1. 1.0 c.c.	Milk
2. 1.0 c.c.	$\frac{1}{10}$
3. 1.0 c.c.	$\frac{1}{100}$
4. 1.0 c.c.	$\frac{1}{1000}$
5. 1.0 c.c.	$\frac{1}{10000}$

The production of acid and gas in each of a pair of tubes is noted as signifying, in the portion examined, the presence of lactose fermenting bacilli. The identity or non-identity of these with *Bact. coli* is unimportant, since their source (the fæces of the cow) is similar. A positive result up to, and including, the two tubes numbered 5, is reported as lactose-fermenting bacilli present in  $\frac{1}{10000}$  c.c. With good quality milks, cultures need be made only from the first 3 or 4 tubes.

Since it has become an official method of milk examination, both in Great Britain and in Ireland, some account must be given of the methylene blue reduction test, although this is not really a bacteriological test and in our opinion, can scarcely be considered even scientific.

For the test, special methylene blue is required. A suitable preparation is "Jensen's Reduktase" tablets, prepared by Messrs. Blauenfeldt and Tvede of Copenhagen. One tablet is dissolved in 200 c.cs. of cold sterile glass-distilled water in a sterile flask. When solution is complete, it is diluted with sterile glass-distilled water to make 800 c.cs.

Test-tubes (6"  $\times$   $\frac{5}{8}$ ") are sterilized by autoclaving and rubber stoppers to fit these are sterilized by boiling for five minutes.

The milk to be tested is thoroughly mixed and 10 c.cs.

are measured into a tube. To this is added 1.0 c.c. of the methylene blue solution and the stopper is fitted to the tube. The dye is incorporated with the milk by slowly inverting the tube twice.

Control tubes are set up. One contains 10 c.cs. of mixed milk and 1.0 c.c. water, the other 10 c.cs. of mixed milk and 1.0 c.c. of the methylene blue solution. Both are stoppered and immersed for three minutes in boiling water to destroy the reducing substances present.

All tubes should be immersed to below the milk level in a water bath provided with a lid (to keep the tubes in the dark) and maintained at 37-38° C.

The tubes should be inspected every half-hour, those which, on comparison with the controls, show decolorization, being removed. At each inspection, every tube should be inverted once.

Decolorization may be taken as complete when a blue colour is seen only in the upper or lower 5 mms. of the column of milk. The time required for decolorization is noted.

Decolorization is due to the combined action of reducing substances naturally present in raw milk and the reducing activity of living bacteria in the milk.

A milk considered suitable for human consumption should not decolorize within 4½ hours in summer (5 hours in Ireland) or within 5½ hours in winter (6 hours in Ireland). A milk produced under clean conditions and with a minimum of bacterial contamination requires much more than these periods for decolorization. If, therefore, decolorization is more rapid, this is due to large numbers of bacteria. Unfortunately there is very little correlation between the bacterial counts and the time required for decolorization.

The method of Prescott and Breed enables one, within a short time, to form an estimate of the bacterial quality of a milk. A slide is laid on a piece of paper ruled in square centimetres. 0.05 c.c. of milk is measured with a pipette on to the slide and, with a platinum wire, is spread evenly into a film covering exactly 2 sq. cms. The film is dried in air, treated with xylol to remove the fat and fixed in methyl

alcohol. It is stained with an aqueous solution of methylene blue (not Löffler's, in which alkali is present which loosens the film from the slide). By means of a micrometer slide, a suitable ocular and suitable tube extension, the diameter of the field of the microscope is arranged to measure 0.016 cm. which gives a field area of 0.0002 sq. cm. The film is then examined with this combination and the average number of bacteria seen in a number of fields is ascertained. A clump of bacteria or a chain of streptococci is counted as one since, by the usual plating methods, either would give rise to only one colony. Since the area of the field (0.0002 sq. cm.) is  $\frac{1}{10000}$  of the whole film, and since 0.01 c.c. of milk was spread in the film, the number of bacteria per c.c. will be (the average number per field)  $\times 1,000,000$ . This method is rapid, cheap and, on the whole, reliable. Its chief disadvantages are that it does not distinguish living from dead bacteria and that, in good quality milk (with less than 100,000 bacteria per c.c.), a number of fields must be examined before any bacteria are found, and hence the results, with such milk, are liable to be inaccurate.

The ordinary dirt bacteria present in milk cause early souring, but are probably devoid of danger to the adult human being. Among them are, however, although they are not yet definitely identified, those which cause infantile diarrhoea, and it is chiefly on account of these that bacteriological examinations of milk are of importance. The organisms of disease in man may, however, gain access to the milk either from the milker or other person handling the milk (enteric fevers, diphtheria and scarlet fever), or from the cow (tuberculosis, undulant fever, and streptococcal sore throat). The use of Wilson and Blair's medium or tetrathionate broth should render it possible to isolate the enteric bacilli from milk, but success cannot frequently be expected owing to the long incubation period of the enteric fevers, and hence the considerable interval between the contamination of milk and the occurrence of cases of the disease. Diphtheria bacilli may be isolated by the use of one of the tellurite media and *Streptococcus pyogenes* (whether coming from a human source or from

the cow and whether causing scarlet fever or sore throat) by plating on blood agar. Since it is not legitimate to conclude that hæmolytic streptococci found by this means are *Str. pyogenes*, such organisms should be subcultured and tested by Lancefield's method. Only streptococci of Group A. are a serious danger to consumers of milk. Myco. tuberculosis is found by centrifuging, at 4,000 revolutions per minute for twenty minutes, 100 to 200 c.cs. of the milk and injecting the deposit into a guinea-pig. After six weeks the animal is killed and, if characteristic acid- and alcohol-fast bacilli are found in typical lesions, the tubercle bacillus is reported to be present. For the detection of *Br. abortus* in milk, 4 c.cs. of whole milk should be injected subcutaneously into the thigh of a guinea pig. Some blood should be removed after three weeks and again after six weeks and the serum tested for agglutinins acting on *Br. abortus*. A titre of  $\frac{1}{40}$  may be taken as establishing the presence of *Br. abortus* in the milk. The animal should be killed in two months and cultures attempted from the lymphatic glands draining the area of inoculation and from the spleen. The best medium to use is agar containing 3 per cent. of glycerol and 10 per cent. of ox or horse serum. Cultures should be incubated for seven days in an atmosphere containing 10 per cent. of  $\text{CO}_2$ .

The results obtained in the bacteriological examination of milk vary enormously. It is impossible under working conditions to obtain sterile cow's milk, but it is quite possible to get milk containing not more than a few hundred bacteria per cubic centimetre. If precautions to prevent access of cow's fæces to the milk are omitted, if improperly cleaned vessels are used, and if the bacteria thus added are encouraged to multiply by keeping the milk for too long a time at too high a temperature (for milk is an excellent culture medium), we need not be surprised to find the total number of bacteria 80 millions per cubic centimetre, and lactose-fermenting bacilli present in  $\frac{1}{1000}$  c.c., as we may occasionally discover in milk bought in a city in the summer.

One of the most important results achieved by the bacteriological examination of milk is in tracing the influence of each

factor in its production and distribution on its bacterial content—the method of milking, cleanliness of cow, nature of pail and sterilization of vessels.

Milk which has been properly pasteurised should not contain more than 30,000 living bacteria (*i.e.* should not develop more than 30,000 colonies) per c.c. and lactose fermenting bacilli should not be present in 1.0 c.c.

Other fluids may be examined in a manner analogous to that employed for water or milk, the extent of the dilution depending on the probable number of bacteria present. Sewage, for example, contains very much larger numbers of bacteria than does water and therefore the dilutions must be carried much further.

**Shellfish.**—Shellfish are examined to determine whether they are likely to convey the organisms of intestinal diseases to man. Attention is directed chiefly to the presence of *Bact. coli*, since this organism is not present in shellfish taken from uncontaminated beds. The method of Klein, somewhat modified, is the one most commonly used. From each batch of shellfish a sample of ten is taken for examination. The shells are thoroughly scrubbed, rinsed with sterile water and opened, using precautions to prevent contamination. The flesh is minced and mixed with the shell liquor and a volume of the resulting fluid from each shellfish is added to a tube of lactose bile-salt broth. In the case of oysters the volume cultured is 0.2 c.c. and with mussels 0.1 c.c. After 24 hours' incubation the tubes are examined and production of acid and gas is presumed to denote the presence of *Bact. coli* in the volume cultured. A positive result is assumed to correspond to the presence of more than 200 *Bact. coli* in the shellfish, and where this number is present the shellfish is classed as "not clean." Out of the ten shellfish not more than four should contain this number of *Bact. coli*. If five or six are so classed "not clean" the batch is regarded with suspicion, while seven or more not clean are sufficient to lead to the condemnation of the batch. By standardizing the volume of each shellfish at 25 c.cs. in the case of mussels, as can be done by the addition of sterile saline, by the inocula-



tion of three volumes from each shellfish (0.5, 0.1 and 0.02 c.c. for mussels) and by the use of two tubes of lactose bile broth with each volume, much more reliable results may be obtained. It is probable that the incubation of cultures at 44° C., in place of 37° C., would prevent the unjust condemnation of shellfish as "not clean," as the presence of *Bact. aerogenes* in shellfish is of much less serious import than the presence of *Bact. coli*.

## CHAPTER XI

### ANTISEPTICS, DISINFECTANTS : CHEMOTHERAPY

A SUBSTANCE, the presence of which in a suitable culture medium prevents the growth of bacteria, is called an antiseptic. If the bacteria are actually killed, it is known as a germicide or disinfectant. Practically all disinfectants act in more dilute solutions as antiseptics, but not all antiseptics, when the concentration is increased, are disinfectants.

The exact mode of action of disinfectants is by no means certain. Some seem to owe their activity to their intense oxidizing power, others produce a coagulation of the bacterial protoplasm, while a third group act as poisons, probably by combining with the protoplasm. Blood, owing to the presence of both specific and non-specific substances, is an excellent germicide when fresh. The number of substances capable of acting as disinfectants is very large. Among these may be mentioned: acids, particularly the mineral acids; alkalies, such as quicklime; oxidizing agents, such as hydrogen peroxide and the permanganates; reducing agents (sulphurous acid); many of the salts of the heavier metals, particularly of mercury, silver and copper; the halogens and certain of their compounds (bleaching lime, iodine, chloroform); formaldehyde, phenol, the cresols, certain dyes and other coal tar derivatives, some volatile oils and some other organic substances.

The greater number of these act best or only when in solution, and, in many cases, their activity varies with the dissociation which occurs in solution. Many factors must be considered in connection with disinfection, of which the chief are: the nature of the disinfectant, its concentration and

the medium in which it acts, the time during which the bacteria are exposed to its action and the temperature, the type of bacteria and the number present. In general, strong solutions are more efficient than weak. Action is quicker on bacteria suspended in water than on those in an albuminous fluid. The higher the temperature the more rapid is the destruction of bacteria. In the case of phenol the velocity of destruction increases eight times for a rise of temperature of  $10^{\circ}$  C. Within limits, lowering the concentration may be balanced by increasing the time of action. Doubling the concentration of mercuric chloride halves the time taken for sterilization, while doubling the concentration of phenol diminishes it about 64 times. Certain organisms are more easily killed by a given disinfectant than are others. Spores are always much more difficult to kill than is the vegetative form of the same organism.

The potency of an antiseptic may be tested by adding it to a series of tubes of broth in such a way that the concentration of the substance in each tube is known. All are inoculated with the organism against which the test is to be made and then incubated. When examined later, we can easily observe the tube with the lowest concentration of the substance in which no growth has taken place. Our result is, however, only applicable to the organism tested, medium used, and the temperature of incubation. If we found in a particular experiment that a concentration of  $\frac{1}{100000}$  inhibited growth, we are not in a position to state that the substance in a concentration of  $\frac{1}{100000}$  will prevent the growth of all organisms in any medium. If, as a practical matter, we are asked how much ammonium fluoride we must add to lemonade to prevent any loss due to bacterial growth (a procedure which, we may remark, is now illegal in Great Britain and Ireland), we must answer only after experiment, using the lemonade in question and a variety of organisms and moulds such as might gain access to the liquid during preparation.

Various methods have been introduced for finding the disinfecting power of substances. Of these the Rideal-Walker

method (the carbolic acid coefficient of the disinfectant) or one of its modifications is most used. In this the potency of the substance against *Bact. typhosum* is compared with that of phenol, which is used as a standard. Two dilutions of phenol ( $\frac{1}{90}$  and  $\frac{1}{100}$ ) are made up and three dilutions of the disinfectant to be tested. All these dilutions are made in water and of each of the solutions 5.0 c.cs. are taken in sterile tubes, which are placed in a rack. 0.2 c.c. of a 20-hour broth culture of *Bact. typhosum* is added to the first tube and a similar amount to each of the others, allowing 30 seconds to elapse between each addition. Thirty seconds after the culture has been added to the last tube, a loopful of the first is added to a tube of broth and each of the others is cultured in the same way, 30 seconds elapsing between each. In all six cultures are made from each tube containing disinfectant, and the arrangement is such that these cultures have been made at intervals of  $2\frac{1}{2}$ , 5,  $7\frac{1}{2}$ , 10,  $12\frac{1}{2}$ , and 15 minutes after the addition to the disinfectant of the culture of *Bact. typhosum*. What we have done is to expose that organism to various dilutions of phenol and of the disinfectant tested for the times mentioned. After labelling, the tubes are incubated, and subsequently a note is made of those tubes in which growth has occurred. In the following table the result of such an experiment is given ("+" indicates growth and "-" the absence of growth in the broth inoculated with the culture, after exposure to the disinfectant in the strength and for the time stated).

Disinfectant.	Dilution.	Time of Exposure in minutes and result in broth.					
		$2\frac{1}{2}$ .	5.	$7\frac{1}{2}$ .	10.	$12\frac{1}{2}$ .	15.
Phenol	$\frac{1}{90}$	+	-	-	-	-	-
	$\frac{1}{100}$	+	+	+	-	-	-
X	$\frac{1}{1200}$	+	+	-	-	-	-
	$\frac{1}{1300}$	+	+	+	-	-	-
	$\frac{1}{1400}$	+	+	+	+	-	-

From this it is seen that phenol  $\frac{1}{100}$  gives the same result as X  $\frac{1}{1300}$ . The carbolic acid coefficient (or Rideal-Walker coefficient) of X is then  $\frac{1300}{100} = 13$ . Where nothing is

known of the disinfectant, it may be necessary to make several preliminary experiments with widely spaced dilutions (*e.g.*  $\frac{1}{100}$ ,  $\frac{1}{500}$ ,  $\frac{1}{1000}$ ) and subsequently to narrow these down when the active concentration is roughly found. It is advisable to make several tests and to average these. Considerable differences might be produced in the coefficient if a concentration of X is found which gave the same result as phenol  $\frac{1}{90}$  and another which agreed with phenol  $\frac{1}{100}$ . The coefficient cannot therefore be taken as more than a rough guide to the efficiency of the disinfectant. Care should be taken always to work at a constant temperature, as may be done by immersing the tubes in a water-bath at 20° C.

Since some disinfectants are exceedingly potent antiseptics, care must be taken against transferring to the broth sufficient of the solution to exercise any inhibiting effect on growth. Among the methods formerly used to test germicides was one in which a thread was steeped in a suspension of the bacteria, dried, and then exposed to the disinfectant solution. Before cultures were attempted it was essential to eliminate all trace of that substance, either by thorough washing or by transforming it into an inert body. In the case of the mercurial disinfectants, which are very potent antiseptics, this was done by treating with ammonium sulphide, which formed, with the mercury, the insoluble sulphide.

The Rideal-Walker method has been very useful in giving us definite information as to the disinfecting powers of various substances; but the value of a disinfectant cannot be judged from the coefficient alone, because that is a measure of its power when acting on a particular bacterium suspended in water. The mercurial salts (*e.g.* mercuric chloride) are powerful disinfectants, but are almost useless in the presence of protein substances, such as blood, pus, and faeces, owing to the formation of insoluble compounds of mercury and albumin. Under similar conditions those substances which owe their activity to the liberation of nascent oxygen (hydrogen peroxide and potassium permanganate) are also comparatively worthless as they are rapidly reduced. On the other hand phenol, the cresols (and preparations containing

them, such as creolin and lysol) and silver salts are little affected by the presence of organic matter, while flavine may even be more efficient in the presence of serum. In order to get over this difficulty, the testing method has been modified by adding to the solution of the disinfectant blood, serum, sterilized fæces or yeast, so reproducing to some extent actual working conditions. Another objection to the Rideal-Walker method is that the organism used is *Bact. typhosum*. A particular disinfectant may be very potent against this organism and, in the same concentration, almost inert against staphylococci or streptococci.

Outside the body disinfectants are of the highest importance in destroying bacteria. For this purpose we must, as a practical matter, consider the question of cost, and from it, combined with the carbolic acid coefficient, we can compute a cost coefficient for the substance. It is obvious that if a given disinfectant has only half the potency of phenol, but costs only one-tenth its price, it is, other things being equal, to be preferred to phenol.

For the sterilization of the skin prior to operation, acriflavine, brilliant green and crystal violet are all superior to tincture of iodine and picric acid which are still commonly used. While these kill surface bacteria, they have but little effect on organisms in the glands and follicles. For hand-rinsing after contamination, 5 per cent. Dettol is probably the most useful disinfectant. For laboratory use, to receive contaminated articles, 2 per cent. lysol is very serviceable. Formalin (1 per cent. or less) and phenol (1 per cent.) are commonly used in the laboratory for killing bacteria in cultures. Both are also used as antiseptics, particularly the latter, in vaccines and sera. Chloroform is also employed as an antiseptic and has the advantage of being eliminated by heat. For "crude" disinfection of urine, fæces and sputum 5 per cent. lysol is probably as efficient as any.

In the disinfection of rooms after infectious disease, sulphur dioxide gas (produced by burning sulphur or obtained from cylinders containing the compressed gas) or formaldehyde (obtained by heating paraformaldehyde or by mixing formalin

and potassium permanganate) are frequently employed, but in neither case is the efficiency very great.

The most successful disinfectant for rendering safe a contaminated water is chlorine. Less than one part per million parts of water, either as chlorine gas dissolved in water or as bleaching lime, is sufficient to destroy the majority of pathogenic organisms in one hour.

In dealing with bacteria in the body, the scope of disinfectants is limited, since any substance capable of destroying bacteria is also injurious, to a greater or less extent, to the body cells. The application of a strong disinfectant to an open wound does more harm than good. It often fails to kill all pathogenic bacteria in the wound, but it does cause the death of many cells, and the resulting necrotic material supplies a nidus in which pathogens may lurk and from which, subsequently, they may emerge. Bland fluids, such as saline, are useful in mechanically removing both bacteria and foreign materials. If disinfectants are to be used, they should be such as not to kill the tissue cells of the part and, even more important, the phagocytes on which the body chiefly relies for its defence against bacteria. Among disinfectants which are both efficient and non-injurious, acriflavine must be placed first. This substance restrains the growth of *Str. pyogenes* in a concentration of  $\frac{1}{100000}$ , while leucocytes are uninjured by it in a concentration of  $\frac{1}{1000}$ . It is one of the very few disinfectants the potency of which is not reduced by the presence of blood. For the treatment of wounds, a 0.2 per cent. solution is recommended. Dettol (5 per cent.) is also active and relatively non-injurious. Some organic silver salts (protargol and argyrol) are particularly valuable in dealing with superficial infections of the mucous membranes.

It has been the aim of chemotherapy for many years to discover a chemical substance which, introduced into the body either by the mouth or by injection, would kill infecting bacteria in the tissues. The value of quinine in the treatment of malaria was discovered empirically, and the fact that it did cause the disappearance of malaria parasites from the blood encouraged those engaged in a search for similarly

active substances. The malaria parasite is a protozoon and, until recently, chemotherapy has had its greatest successes in dealing with protozoal disease. Germanin and tryparsamide are undoubtedly efficacious against trypanosomes and *v. Hayden* 471 against leishmaniæ, but trypanosomes and leishmaniæ are protozoa.

Prior to 1935, the only chemical substances which had been proved to be capable of killing bacteria in the body were salvarsan, neosalvarsan and similar arsenical derivatives, and these were active mainly, if not entirely, against spirochætes which are near allies of the protozoa.

The discovery of Prontosil inaugurated a new era in chemotherapy. From it have sprung a bewildering number of new drugs with an even more bewildering variety of names. For convenience we propose to refer to them as the sulphanilamides.

Clinical trials, supported by laboratory tests, have proved that these compounds are capable of freeing the human tissues from many types of pathogenic bacteria. Among these are streptococci, pneumococci, meningococci, gonococci and *Bacterium coli*. It will take several years before the powers and limitations of the members of the group are completely revealed but, in the few years since the discovery of Prontosil, we have learned something of their mode of action. Some of them are disinfectants: that is, they are capable of killing bacteria when tested *in vitro* against suspensions of the bacteria in broth or saline. But, for this purpose, it is usually necessary to have concentrations higher than can be attained with safety in the body. In very much higher dilutions, such as the body can tolerate, they are antiseptic or bacteriostatic, that is they prevent the growth of the organism in broth without actually killing it. This bacteriostatic power may be easily demonstrated when the number of bacteria is small: with very large numbers the substance fails to arrest growth. When similar tests are made in defibrinated blood instead of in broth, the bacteria, if not present in overwhelming numbers, are killed. From such experiments it is fairly certain that, in the body, these sub-



stances act mainly by preventing the multiplication of the bacteria against which they are active. The bacteria are then destroyed in the usual way by antibodies and leucocytes.

For the treatment of a bacterial infection to be successful, we must know the type of infecting organism in order to select the appropriate sulphanilamide: we must administer this substance in a dose large enough to be effective and yet not so large as to be toxic: the patient must possess some degree of resistance to the organism. The drug, in a concentration which the body tolerates, merely holds the bacteria in check: the normal defence mechanism of the body, whether cellular or humoral, is required for their elimination. The humoral defence may be the result of the development of an active immunity by the patient during the course of the disease or may be assisted by passive immunization with antiserum.

An understanding of these principles is essential for the successful employment of the sulphanilamides in practice. A blind administration of them to every patient with a high temperature is not, now, likely to discredit the drugs so much as the practitioner. The combined action of drug and antibodies explains why, according to some authorities, administration of one of these substances in a case of gonorrhœa is best delayed until some degree of active immunity has developed and why the combined administration of sulphanilamide and antiserum in cerebro-spinal fever and pneumonia is more successful than either alone.

Probably the most successful chemotherapeutic agent, apart from the salvarsan and sulphanilamide groups, is mandelic acid in urinary infections. The use of this drug is based on sound bacteriological knowledge—that such an organism as *Bact. coli* cannot multiply if the pH of the medium is too low. By the administration of mandelic acid or some substance which produces it, the reaction of the urine is rendered so acid that the bacteria are killed.

Research in chemotherapy requires the co-operation of the chemist, the bacteriologist and the clinician. *In vitro* tests of a chemical substance on a particular bacterium are often of little assistance in assessing its possibilities in treat-

ment. In the body, the substance may be either more or less active than in the test-tube, and so, extensive animal experiments are required. If the substance passes these successfully, it must be tested cautiously in the human body to demonstrate that it is not, in the required dose, dangerous. Only then may it be submitted to extensive clinical trial, on which its reputation ultimately depends.

## CHAPTER XII

### BACTERIA IN HEALTH AND DISEASE

THE first lesson in bacteriology which it is essential that the student should learn is that bacteria are present almost everywhere. He may assume, and he will rarely be wrong, that the air around him, the water he drinks, the food he eats, the surface of his body and everything he touches are all contaminated with bacteria unless specific measures have been taken to destroy them.

Bacteria may be divided into two classes: saprophytic (living on dead organic matter) and parasitic (living on or in the animal body). The parasitic bacteria may be further subdivided into three subgroups—strict pathogens, facultative pathogens and non-pathogens. It cannot be argued that on this, or any basis, the lines of division between the various groups are clear-cut. The introduction into the body of large numbers of saprophytic bacteria may give rise to illness: the majority of parasitic bacteria can be cultivated in artificial media: apparently perfect health may persist when bacteria, universally recognized to be strictly pathogenic, exist in or on the body. The facultative pathogens (which Theobald Smith happily described as "opportunists") commonly exist on the surface of mucous membranes and apparently do no harm, but when introduced into the tissues may be capable of causing disease. It is probable that all pathogenic forms originally arose from adaptations of saprophytic bacteria to a parasitic life, but experiments designed to show such a change have, for the most part, failed. So long as their limitations are realized, the terms saprophytic, parasitic, and pathogenic are convenient and may be employed.

Any investigation into the bacteriology of a morbid condition requires a knowledge of the normal bacteria of

the portion of the body studied, and we must, therefore, devote some attention to the bacterial flora of the human body.

The exposed portions of our bodies are constantly receiving bacteria from objects with which they have come in contact, but the majority of these do not grow and so each part maintains its own particular flora. Staphylococci and diphtheroid bacilli are almost universally found on the human skin. The same bacteria are usually present in the external ear. The conjunctival sac has considerable disinfecting powers so that, unless it is diseased, few bacteria are to be found in that situation. It is, therefore, wise, before an operation on the eye is performed, to culture a conjunctival swab on blood agar and to postpone operation if streptococci, pneumococci or *Staphylococcus pyogenes* (aureus) are found: diphtheroid bacilli may be ignored as may also *Staphylococcus pyogenes* (albus) if their numbers are small.

The nostrils serve as a filter for the inspired air, and so the bacteria of this region represent the bacteria of the air and include many varieties of cocci and bacilli together with yeasts and spores of fungi. The further we proceed from the exterior, the fewer and more constant are the bacterial species encountered. About the throat, in the presence of no obvious signs of disease, streptococci of the non-hæmolytic varieties, cocci of the *N. catarrhalis* type and staphylococci very commonly occur, while pneumococci and Friedländer's bacillus are not infrequent. The deeper portions of the respiratory tract, the finer bronchioles and the alveoli of the lung, are normally sterile.

In the mouth the presence of the teeth acts as a complicating factor since it is doubtful if these, in the adult, are ever completely healthy. About their junction with the gums we commonly find non-hæmolytic streptococci, *Leptothrix buccalis*, various spirochætes and *L. odontolyticus* which is, by some, described as the cause of caries, now so common as to be almost a normal condition.

The alimentary tract presents a highly complicated flora and, a point which can hardly be over-emphasized, one which

is comparatively easily altered by changes in diet. The contents of the healthy stomach are practically sterile, as are also those of the duodenum, owing to the presence of hydrochloric acid in the gastric secretion, but the intestine contains an enormous variety of bacteria. It has been calculated that an adult excretes in the fæces about  $3 \times 10^{13}$  bacteria daily, the majority of which are dead. The most numerous are *Bact. coli* and other Gram negative bacilli, including *Proteus vulgaris*, *Bact. aerogenes*, and *Ps. pyocyanea*. In addition, non-hæmolytic streptococci, *L. acidophilus*, *B. mesentericus* and anaerobic spore-bearing bacilli (chiefly *Cl. welchii*) are almost always present. The relative proportions of the various organisms are subject to wide variations, and it is fallacious to ascribe any ætiological importance to quite wide departures from the normal, although this is frequently done. By the consumption of sour milk, together with excess of lactose, the intestine may be implanted with *L. acidophilus* and the fæcal picture transformed from one predominantly Gram negative to one having the majority of the bacteria Gram positive. The closely related organism, *L. bulgaricus*, cultures of which are frequently regarded as a cure for "auto-intoxication," a condition the very existence of which is doubtful, does not become implanted in the intestine as does *L. acidophilus*. The fæces of breast-fed infants contain chiefly Gram positive bacilli, the most important of which are *L. bifidus* and *L. acidophilus*.

About the external genitals, in both sexes, various Gram positive cocci, *Bact. coli*, smegma bacilli and spirochætes are found. Few bacteria exist in the urethra and the urine in the bladder is sterile in health. In the vagina, another region of doubtful normality, the reaction is distinctly acid, and Döderlein's bacillus, an organism related to, if not identical with, *L. acidophilus*, is common; *Bact. coli*, fæcal streptococci and many other varieties of bacteria can, however, exist there without obvious morbidity. The interior of the uterus and tubes is sterile.

This brief review will serve to indicate the numbers and varieties of the bacteria which are human parasites or, to use

what is possibly a better term, commensals. As has been indicated, some of these, although of very common occurrence, may be the cause of slight departures from the state of perfect health but, at least, their presence is compatible with the absence of definite disease. Since they live on the surface, either actual or physiological, of the body they are termed parasites, but their existence is really a saprophytic one, for they consume the secretions, excretions and waste products of the body without inflicting any harm upon it. A highly poisonous substance, so long as it is enclosed in its bottle, is only potentially dangerous to life but, when swallowed, its potentiality to harm the body becomes an actuality. So *Bact. coli* in the intestine and streptococci about the mouth are to be regarded as potentially dangerous since, as a result of imperfect nutrition, exposure to cold, interference with blood supply and other conditions, not yet thoroughly understood, they may invade the body and initiate in the one case cystitis, pyelitis or abscess formation and in the other sub-acute endocarditis or arthritis. Many of the other bacteria which occur normally about the body are potentially pathogenic; under normal conditions, however, as we shall see, it is efficiently protected against them. It is to be noted that the type of disease produced by this class of organisms is not, as a rule, infectious and, unlike disease due to the frankly pathogenic organisms; does not pass from individual to individual in the community. The parasitic organisms are, in fact, almost exclusively pathogenic for the host on whom they live, and consequently play an important part as secondary invaders in disease due to other causes whether bacterial (*e.g.* tuberculosis of the lungs) or metabolic (*e.g.* diabetes).

We must now leave, for the moment, these facultative pathogens in order to consider the other and more important class, the strict pathogens. We leave them physiologically outside the body; the methods by which they gain entrance thereto will be considered later.

The strict pathogens appear to have as their chief function the production of disease in man and animals. When they

reach a suitable host they multiply in his body, producing in it their characteristic disease and escape to the body of another victim. We may first consider the methods by which these organisms spread from host to host and the route by which the body is invaded. In temperate climates the majority of epidemic and endemic diseases spread directly or indirectly from individual to individual. The infecting person may himself be suffering from the disease or he may have recovered from it and yet still harbour the causative organism, in which case he is a "carrier." The carrier condition is always present during convalescence but is not usually of long duration, the organism being eliminated soon after health has been regained. In some cases, however, the organism, normally pathogenic, may become merely parasitic on the particular individual while retaining its pathogenic properties for others. He is no longer harmed by its presence because his body has been educated to neutralize its injurious products, but he is a danger to others. The carrier state may continue for years and, owing to it, many epidemic and endemic diseases—typhoid fever and diphtheria for example—are difficult to stamp out. It occasionally happens that a strict pathogen may be present in the body of an individual for some considerable time before the disease develops. In such cases it is possible that all the conditions necessary for invasion have not yet been fulfilled. Such a person is spoken of as a healthy carrier and is a danger to others as well as to himself.

The bacteria causing many of the commonest infectious diseases, such as diphtheria, scarlet fever, measles and tuberculosis, depart from one host and enter another by the respiratory tract. In quiet breathing the expired air is sterile, but in speaking, coughing, and sneezing droplets of saliva and sputum are sprayed out from the mouth and nose, and these may contain pathogenic bacteria. Droplets may travel through the air for considerable distances—several yards for example—before falling to the ground, or the water which they contain may evaporate leaving bacteria, together with proteins and salts, as droplet nuclei which may remain sus-

pended in the air for hours. So, either by droplets or by droplet nuclei, bacteria gain their transfer to a fresh victim. Conditions of over-crowding and lack of adequate ventilation render transfer in this way particularly likely. Larger drops may fall to the ground and become dry, and the bacteria may lie in the dust, for a longer or shorter time, until stirred up by wind or a misdirected desire for cleanliness which expresses itself by dry-sweeping of the floor. Then the bacteria are inhaled with the dust and another victim secured.

The next group of diseases are those (such as the enteric fevers) affecting chiefly the intestinal tract. The causal organisms leave the body either with the fæces or, more rarely, the urine. In order to cause fresh infection they must be swallowed. Formerly these diseases were broadcast chiefly by water supplies, to which the infected excretions gained access; and to a less extent, by food, particularly milk and shellfish. Public health knowledge and legislation have been more successful in combating the intestinal than the respiratory group of diseases, chiefly because the disposal of excreta and the protection of water and food are matters fairly easily arranged. To-day such diseases are spread largely by the neglect of carriers to wash their hands after defæcation and by the filthy habits of flies which feed on excreta and transfer the organisms to food.

The third group of diseases are those normally requiring direct or indirect contact between the person harbouring the organism and the one about to be infected. Among diseases in which contact is usually direct the outstanding examples are syphilis and gonorrhœa; they are diseases called, quite correctly, contagious. Examples of indirect contact are the pencil sucked alternately by the diphtheria carrier and his school-fellow, and the midwife's hands which convey the organisms of puerperal sepsis from case to case.

The next group, a very important one, consists of the diseases in which the organism is transferred from individual to individual by the intermediary of a blood-sucking insect. Typhus, spread by the louse, malaria by the anopheles mosquito, and sleeping sickness by the tsetse fly, will serve as examples.



Lastly, we have to consider those diseases which occur in the lower animals as well as in man and are commonly spread from the former to the latter. Rabies from the dog, anthrax from the sheep and glanders from the horse are sufficient to demonstrate the importance of this group.

It is not pretended that the above is a complete account of the various methods by which organisms of disease reach the body of man ; it is only a brief summary of the more important of them. It enables us, however, to visualize how the strict pathogens make contact with their human victim. We have already seen that the facultative pathogens are ready, on the surface of the body, to invade the tissues when circumstances are favourable. How does man, surrounded by so many dangerous enemies, continue to escape their activities? The answer to this question will be deferred to later chapters, in which will be considered man's defences against bacteria, that is, his immunity. Here we are considering the bacteria, and we may now suppose that bacteria have passed his first line of defence and succeeded in invading his tissues. But mere invasion is not synonymous with infection. Many types of bacteria may invade, comparatively few can infect, and so we must consider, from the point of view of the bacteria, how a successful invasion can become a definite infection.

In order that infection may follow invasion certain essentials must be fulfilled. The organisms must be virulent ; they must be present in sufficient numbers ; they must invade the body by the appropriate avenues ; they must be able to resist the defensive forces of the host ; the host must be susceptible. Each of these factors must now be considered.

1. **Virulence.**—By the virulence of a bacterium we mean its power of producing disease. This depends on the organism's aggressiveness and on its toxicity. Aggressiveness is the power of multiplying in the body, toxicity is the power of producing poisonous substances. The two are to a considerable extent independent. *Cl. tetani* and *C. diphtheriæ* are strong toxin producers with but little aggressiveness. *B. anthracis* is only slightly toxic but is remarkably aggressive. Toxicity is a characteristic of a bacterium which remains

constant for long periods, but aggressiveness is variable. It is easily lost in artificial culture, in some cases owing to the loss of capsules, in others owing to the development of rough variants, and in yet others from quite unknown causes. It may, on the other hand, be increased by animal passage, that is, by transfer from one animal to another. The explanation of this heightening of virulence is probably that such treatment selectively encourages the few highly virulent forms in the culture used. These, being virulent, grow readily in the animal's tissues, while the avirulent tend to disappear, so the material removed contains a higher proportion of virulent bacteria than that introduced. There is a similar increase of virulent forms at each transfer until maximum virulence is attained, when all the bacteria are virulent. Aggressiveness is something which we find hard to explain and for which the reasons are obscure. We know that foreign substances (dead tissue, calcium salts, quinine or other organisms in the case of the anaerobes, and silica in the case of the tubercle bacillus) may act as adjuvants and increase aggressiveness. Bail gave the name "Aggressin" to a hypothetical substance which he believed was produced by bacteria in the body but not in artificial culture. He demonstrated that the washings of the peritoneum of an animal which had died as the result of an intraperitoneal injection, when freed from bacteria, were capable of making a sub-lethal dose of that organism fatal for a new animal, although the washings containing aggressin were without effect when introduced alone. It is possible that aggressin acts largely by preventing phagocytosis of the invading bacteria. The washings may, for example, contain leucocidin, which destroys leucocytes and so must increase the aggressiveness of the organism. They probably also contain bacterial substance which can combine with antibodies present and so divert them from acting on intact bacteria.

2. **Number.**—To produce an infection it is essential that a certain number of bacteria (depending on the type of organism, its virulence and other factors) must invade the tissues. It has been found experimentally that a single anthrax bacillus can cause a fatal infection in 28 per cent. of

mice, while 10 anthrax bacilli are sufficient to kill every mouse inoculated. The dose of bacteria taken into the body by the mouth is of importance. When 1,000 *Bact. typhi-murium* are administered to each of a number of mice by this route, 15 per cent. of the mice die; when the dose is 100 millions, 49 per cent. of the mice die. With less virulent organisms a large number invading the tissues at the same time will probably cause an infection, but if the number of invaders is small they are destroyed and some degree of immunity results. Infection is most likely to occur when the organisms are being received in large numbers and constantly. Small numbers received intermittently will probably be destroyed. In many respiratory diseases infection is determined by the proximity to a source of the bacteria and by the time of exposure. So epidemics of respiratory diseases frequently originate in barracks and dormitories. During the day proximity is less close and exposure for shorter periods and, therefore, infection is less probable. The best prophylactics against diseases of the respiratory tract are absence of overcrowding, efficient ventilation and open air conditions at intervals.

The virulence and the number of invading bacteria are closely related factors. Virulence of a particular strain of an organism is estimated by the "minimum lethal dose," the volume of a broth culture, for example, which must be introduced into each of a number of experimental animals in order to kill 75 or 100 per cent. of them. The minimum lethal dose, owing to variation in resistance of different animals, cannot be accurately estimated with a small number of animals; large numbers must be employed if even approximately accurate results are required. When virulence is high, an exceedingly minute dose may lead to infection; when virulence is low, the dose must be larger.

3. **Avenue of Infection.**—This is occasionally of considerable importance, especially for those organisms of slight aggressiveness. In some cases an organism can only produce its characteristic disease by acting on some definite tissue in the body. The cholera vibrio and dysentery bacilli act almost

entirely on the intestinal tract and, when injected subcutaneously, do not produce intestinal disease. The gonococcus can enter the body only by the external genitals or the eye.

4. **Defence of Bacteria.**—When bacteria are attacked by the protective agents of the body they are not passive. One method of defence often used by bacteria is to produce something which destroys the leucocytes. Another is to protect themselves by the development of capsules. Probably a still more important method of resistance is the production of alternative forms, not acted upon by the host's antibodies. The spirochætes of relapsing fever offer an excellent example of this. At the time of crisis the serum of the patient is actively destructive to the existing spirochætes, and yet he may suffer from a series of relapses. In each of these is found a serologically distinct strain of spirochæte produced by the few survivors, and these are not acted upon by antibodies already developed. The trypanosomes (although not bacteria) illustrate another defence of the parasite. They are easily killed in the animal body by certain arsenical preparations but, if the dose of the drug administered is too small to eradicate them completely, a few survive and their descendants can resist much larger doses of the same drug. Even if these are passed through a long series of normal untreated animals they retain this drug-fast characteristic. Nature, in the animal's body, is constantly forging new weapons against bacterial invaders, but she is quite impartial and, in the bacteria, is similarly hard at work, devising means of rendering useless these very weapons.

5. **Susceptibility of Host.**—Susceptibility is the reverse of immunity; when one is high, the other is low. The question will be considered in greater detail in the chapters on immunity.

When these five factors, which we have considered, are in favour of the invading bacteria, infection occurs and the bacteria grow in the tissues of the host. It is natural to ask

how do they injure the host, how do they cause disease? Bacteria in the body absorb food materials destined for the nourishment of the host's tissues and, by accumulation, act mechanically as foreign bodies. It is not likely that in either of these ways serious damage is inflicted. More commonly they do harm by the production of poisonous substances of which various types are known.

1. Toxins.—Formerly a sharp distinction was drawn between two classes of toxins—exotoxins and endotoxins. The former were believed to be secretions of the growing bacteria, as they were found in filtrates of broth cultures: the latter were supposed to be contained within the bacterial bodies and to be liberated only by their dissolution. We do not now believe that all exotoxins are secretions for, in some cases (*Cl. tetani* and *C. diphtheriæ*), little toxin is found during the phase of active growth, and it may increase markedly while the number of living bacilli is decreasing, and while autolysis is in progress. The two terms can, however, be conveniently retained so long as it is realized that there is no very sharp dividing line between them. In general, exotoxins are active in very minute doses, endotoxins require large doses to produce their effects; exotoxins cause very characteristic effects on animals, and may act only on certain tissues, endotoxins tend to be much less specific; exotoxins are usually thermolabile (although some are not), endotoxins thermostable. The most characteristic difference, however, is that exotoxins are strongly antigenic and give rise, in the animal body, to antitoxins, while endotoxins are much less capable of leading to the production of antitoxins. Antitoxins developed against exotoxins can completely neutralize these. If a given volume of antitoxic serum can render non-fatal 100 lethal doses of exotoxin, twice the amount of serum will neutralize 200 lethal doses. With endotoxins, however, while a certain amount of antiserum may pro-

tect against 5 lethal doses of endotoxin, ten times that amount may fail to neutralize even 10 lethal doses. The number of bacteria known to produce definite exotoxins is small, but newer cultural methods have revealed the production of typical exotoxins by some organisms formerly believed not to produce them (streptococci and staphylococci). It is probable that endotoxins are the immediate causes of the diseases produced by the majority of pathogenic bacteria.

2. In the case of many bacteria no exotoxins that can be demonstrated are produced in artificial cultures, nor are the bodies of the killed bacteria very toxic when injected and yet the patient displays all the signs of toxic absorption. It has been suggested that many of the pathogenic bacteria (*e.g.* *B. anthracis*) are capable of producing toxins in the body of the infected animal but not in artificial culture.
3. Bacterial protein.—When bacteria in the body disintegrate their protein is free and is injurious to the tissues which react to the presence of any foreign protein. It is possible that many of the general symptoms of infectious diseases, such as fever, are due to bacterial protein.
4. Other poisonous products.—When bacteria live in the body they digest the proteins of the tissues and the body fluids to render them suitable for their nourishment. Digestion products of proteins are frequently highly toxic, and it is probable that those resulting from bacterial digestion are of this nature. In some cases injurious chemical substances, such as lactic and butyric acids, are produced by bacterial action. Further, by the action of the bacteria, body cells are killed and such necrotic material, in many cases, is also toxic. In this way the presence of bacteria in the body can give rise to three further groups of poisonous substances.

From the foregoing it is apparent that bacteria inflict injury

chiefly by producing poisonous substances. These first act locally, giving rise to the primary lesion which, in many cases, is so characteristic that from it the type of infecting organism can be deduced; they then are carried by the blood stream to other parts of the body where they may affect various tissues. In almost all infections the brain centres controlling temperature, heart beat and respiration are affected, and so the signs of fever are produced. Degeneration or even death of cells may occur, either in the nervous system, causing paralysis, or in various organs of the body such as the heart, where the muscle cells are affected, and the kidneys, adrenals and liver, where the secreting cells in particular are injured. Further evidences of the general effects of a primary, local lesion are seen in the alterations produced in the circulating blood (anæmia, leucopenia, polymorphonuclear leucocytosis, mononuclear leucocytosis for example).

The type of local lesion varies with the species of bacterium, its virulence, the susceptibility of the host and the particular tissue of the body affected. The lesion results from the effects of two sets of forces, the destructive forces of the bacteria leading to degeneration and necrosis of cells and the defensive forces of the host. The effect of the latter is often more obvious to the patient and observer. The cardinal signs and symptoms of inflammation—heat, redness, swelling and pain—are due to the host and not directly to the bacteria. An exudate is commonly present which may be fibrinous, catarrhal, hæmorrhagic, membranous or purulent. The local lesion may remain strictly localized (*e.g.* a boil) or may be of a spreading character (*e.g.* cellulitis), the bacteria making their way rapidly through the tissues. They may spread to remote parts of the body by means of such natural channels as the ureters or bile ducts, by the lymphatics or by the blood stream. It is probable that the blood is fairly commonly invaded by bacteria. Usually these are few in numbers, they fail to multiply in the blood and are soon destroyed. To this condition the term bacteræmia is applied. In more serious infections there may be an actual growth of the bacteria in the blood (septicæmia). When a thrombus becomes infected,

small portions containing bacteria may be detached, and may lodge in remote capillaries where they originate metastatic abscesses (pyæmia). The term toxæmia implies that bacterial toxins (without the bacteria themselves) are present in the blood stream.

We have dealt in this chapter with the ways in which bacteria gain access to the body and cause infection. The outcome of an infection, whether recovery or death, depends chiefly on the powers of resistance displayed by the host.

It will be obvious that there are two chief groups of methods by which the spread of infectious diseases may be checked. The first is of a general nature and aims at preventing the causal organisms from being conveyed from cases or carriers to fresh individuals (isolation, disinfection, disinfection, protection of water and food supplies). The second is personal in character and attempts to increase the individual's powers of resistance by some method of immunization.



## CHAPTER XIII

### INTRODUCTION TO IMMUNITY

By immunity we mean the power which the animal body has to resist the action of organic foreign substances, actually or potentially harmful. The term is frequently restricted to resistance against bacteria and their products, and it is with this aspect of immunity that we are chiefly concerned.

#### **Natural Immunity**

In the last chapter we saw that, while the majority of bacteria were saprophytes, quite innocuous to living animals, some were strict or facultative pathogens, capable of producing disease once they had entered the body. We must now consider the defences of the body which tend to keep bacteria from entering the tissues. The chief of these is the integrity of the investing layers of epithelium—skin and mucous membrane. So long as these are intact, they offer an almost complete barrier to the passage of bacteria. It is, therefore, chiefly by trauma that bacteria can effect an entry into the body. The degree of trauma necessary varies with different bacteria; it must be extensive in the case of tetanus, but the very slightest suffices in syphilis. Next we have the various secretions and excretions which bathe the skin and mucous membranes—sweat, tears, mucus, saliva, gastric and intestinal juices and urine. These act mechanically by washing off bacteria which have adhered to the surface and, in the case of some of the mucous membranes, are aided by the action of cilia, which create currents directing bacteria away from deeper structures. Of these fluids the most important is probably mucus. In many cases mechanical action is assisted by the presence of a substance—Lysozyme—

which is capable of killing and dissolving certain varieties of bacteria. This substance is found in all the body secretions, except urine, and also in the tissues. The reaction of some of the secretions is a matter of considerable importance, particularly the high degree of acidity of gastric juice, which almost completely destroys bacteria in the stomach. If the secretion of hydrochloric acid ceases or is diminished, there is a great increase in the number of bacteria found in that organ and also in the duodenum. The acidity of sweat and the presence in it of lysozyme serve to explain the power of the skin to kill bacteria which may lodge on it.

There is reason to believe that diet plays some part in these largely mechanical means of defence. It is found that lack of vitamin A increases the liability of experimental animals to bacterial disease, chiefly of the respiratory and alimentary tracts. This is probably due to alterations in the mucous membranes, whereby they become more permeable and less active in secreting mucus. It has also been observed that guinea-pigs fed on a diet which keeps them close to the scurvy line are particularly susceptible to infection by the pneumococcus.

Despite the barriers outlined above invasion does occur, probably quite commonly, but every invasion does not lead to an infection. Once the skin or mucous membrane has been passed the bacteria find themselves exposed to the action of the tissues and tissue fluids, which contain a variety of substances injurious to them. Lysozyme, which has already been mentioned, is present in practically all tissues, but in largest amount in cartilage, a tissue devoid of blood supply and so cut off from the protective substances of the plasma. Lysozyme acts chiefly on Gram-positive cocci, such as are commonly present in air. In addition to lysozyme three other classes of bactericidal substances are found in tissue fluids—Leukins (derived from leucocytes), Plakins (from blood platelets), and  $\beta$ -Lysins. While these may be distinguished by slight differences in their mode of action and thermostability, such details are of little practical importance. They are all found in the normal body and act chiefly

on the sporing anaerobic bacilli, the proteus group, some Gram-positive cocci, and commonly occurring saprophytes. Lastly, in the plasma, are found Normal Opsonins which act on the bacteria and render these easily phagocytosed by polymorphonuclear leucocytes. Their mode of action will be further considered in a later chapter.

If bacteria have safely passed these dangers, infection may result if they possess sufficient virulence, are present in sufficient numbers and in a suitable tissue, points already considered. Virulence for one animal does not, however, mean virulence for all, and in that lies one of the greatest mysteries of natural immunity. We are really quite ignorant of why syphilis, gonorrhoea, and cholera are exclusively human diseases, or why glanders affects horses and not cattle. We think it possible that, in some cases, this species immunity is due to the unsuitability of the tissues of the non-susceptible animal for the growth of the bacteria, but in others we know that the animal's cells are inert to the action of the toxins of the organisms, as is the case with the rat, which is almost unaffected by very large amounts of diphtheria toxin. Even in the same species there are curious inequalities in susceptibility in different races as, for example, the relative immunity of Algerian sheep to anthrax. Some of these, as the extreme severity of measles in aboriginal tribes, are probably due to the lack of inherited immunity. Lastly, it must be recognized that the various tissues of the body are not equally susceptible to bacterial invasion or to the action of bacterial toxins. Muscles are rarely affected by the tubercle bacillus; diphtheria seldom affects the oesophagus; the skin is particularly susceptible to anthrax; tetanus toxin affects chiefly nerve cells, and the toxin of diphtheria certain epithelial and nerve cells.

Immunity may be considered in two aspects—natural and acquired. Up to the present we have considered only natural immunity; we must now turn to that type of immunity which is acquired, whether by natural means or artificially. It is probable that the natural immunity of one human being is fundamentally the same as that of another human being, except inasmuch as it has been modified by

malnutrition or some factor which interferes with normal health—lack of fresh air and sunlight, fatigue or excesses of various kinds, all of which appear to increase susceptibility to certain bacterial diseases.

### **Acquired Immunity**

Immunity against a particular disease may be acquired in a variety of ways—

#### **A. Actively.**

- (a) By an attack of the disease.
- (b) By repeated exposure to sub-infective doses of the bacteria.
- (c) By some artificial process.

#### **B. Passively.**

- (a) By inheritance.
- (b) By mother's milk.
- (c) By some artificial process.

Acquired immunity is due, fundamentally, to the presence in the body of certain substances which are inimical to the growth of bacteria or which can render harmless their poisonous products and also to altered reactions of certain cells in the body to bacteria or their products.

It will be convenient to consider separately immunity acquired by natural means, A (a) and (b) and B (a) and (b), and immunity acquired by artificial means, A (c) and B (c), but it cannot be over-emphasized that the processes are really the same. Man has merely imitated Nature in order to secure Nature's results without, in some cases, running the considerable risks involved in a too slavish imitation of her methods.

**Naturally Acquired Immunity.**—It has long been known of certain infectious diseases that the overcoming of one attack usually protects the individual for the rest of his life from a second attack. This is true of smallpox and typhoid fever. This immunity is due to the acquisition by the cells and fluids of the body of new powers of attacking the causative organism or, more probably, to an enormous increase of such

pre-existing powers. By overcoming one attack these powers have been acquired or augmented, and usually they persist, to a greater or less extent, for the rest of life. But in some diseases (diphtheria, scarlet fever) it is found that, while young children are susceptible, adults are immune, although they may never have had an attack of the disease. The finding of an increasing proportion of immunes, as age advances, in a population which has almost certainly never been exposed to the presence of the organism in question (*e.g.* the diphtheria bacillus), suggests that the production of apparently specific protective substances may be a physiological process similar to the gradual appearance in the blood of isohaemagglutinins which are not present at birth and which are certainly not produced in response to the introduction, into the body, of the blood cells of another individual. But even if these substances may appear without external stimulus, there is no doubt that frequent exposure of an individual to such a pathogenic organism as the diphtheria bacillus, in small doses, hastens their production. That this is so is shown by the much higher proportion of Schick negatives among slum children than among children of the same age belonging to the "sheltered" classes in the same town. The slum children are frequently exposed to sub-infective doses of diphtheria bacilli with the result that protective substances, similar to those found in the body of a person who has recovered from an attack of diphtheria, are produced in a higher proportion and at an earlier age than in the case of the children of the rich who are rarely exposed to the action of these bacteria.

Diphtheria also furnishes a good example of immunity acquired passively by an infant from the mother. The disease rarely attacks in the first year of life, and it is found that infants have, in their sera, a substance which neutralizes diphtheria toxin and so protects them. This they obtained from their mother's blood through the placenta. In man and some animals the young inherit immunity against certain diseases, to which their mothers are immune, in this way; with other animals there is little, if any, transfer of protective substances from the maternal to the foetal cir-

culatation and, instead, these substances are passed to the offspring in the colostrum and milk. At an early age, and only then, can the substances be absorbed from the alimentary tract into the circulation. Although transfer through the placenta is more important in human beings, there is little doubt that protection is also afforded to the offspring by being suckled, especially for the first few days of life.

**Artificially Acquired Immunity.**—Man's first deliberate effort to protect himself against a disease was by voluntarily exposing himself to infection. In parts of Asia, for several centuries at least, material taken from a mild case of smallpox was inoculated into those desiring protection. The result was, of course, the development of smallpox often, but not necessarily, of a mild type. Since, at that time, almost every one, at some period of his life, was attacked by smallpox, it was thought preferable to run the necessary risk at a selected time rather than be infected by chance. This, although in a sense artificial, was really an example of natural immunity development, since those inoculated underwent the natural disease. The first introduction of artificial immunity was due to Jenner, who discovered that the disease of cattle known as cowpox or vaccinia was transferable to man, and that its occurrence gave subsequent protection against smallpox. It is probable that the organisms of the two diseases were originally identical, and that that of vaccinia has been modified by passage through cattle. The immunity against smallpox afforded by vaccination may therefore be regarded as one produced as a result of the action of a virus modified by passage. A definite disease is produced, but that disease is not variola.

No other advances were made until the work of Pasteur, who laid the foundations of artificial immunity as well as of bacteriology. He discovered methods of modifying the virulence of the living causes of several diseases so as to enable them to be used for the purpose of conferring immunity without serious risk.

There are two types of artificially acquired immunity:  
(1) Active. (2) Passive.

In actively acquired immunity the defensive forces come entirely from the cells and tissues of the subject's body, being called forth as a result of the introduction into the body of the bacteria themselves, living or dead, fully virulent or modified, or else of some product of the bacteria. It must be emphasized that the resistance comes from the man or animal who receives the injections; it is his response to the threat conveyed by the substance injected.

In passive immunity the subject takes no part in the defence. He receives the blood or serum of another animal which has been actively immunized. This fluid contains substances inimical to the activities of the bacteria or their products, and it is these substances in the blood or serum introduced, which confer, passively, immunity on the subject.

Active immunity is produced by—

1. The injection of bacteria—

- (a) Living and fully virulent, but in small, sub-lethal (non-fatal) doses.
- (b) Living but with reduced virulence.
- (c) Dead.

2. The introduction of bacterial products, chiefly toxins, in sub-lethal doses or modified by chemical action.

The introduction of small doses of fully virulent bacteria is dangerous, and is not to be recommended, at least as an initial step, in immunizing an animal. There are many ways of attenuating, *i.e.* reducing the virulence of a given bacterium. Continued culture in artificial medium is frequently sufficient. The pneumococcus, for example, after a few generations in laboratory culture, has lost a great part of its original virulence. Other organisms, such as the tubercle bacillus, retain their virulence for much longer periods; but even the tubercle bacillus, after the lapse of possibly several years, becomes entirely non-virulent. This method was accidentally discovered by Pasteur, who found, on returning from a holiday, that a culture of the bacillus of chicken cholera, which had been very virulent, had now lost its lethal action on fowl, but caused in those inoculated with it the

development of immunity which protected them against infection when they were subsequently inoculated with a fully virulent culture. A slightly different method is also due to Pasteur, who found that the cultivation of *B. anthracis* at a temperature above that of the body (at  $42^{\circ}$  to  $43^{\circ}$  C.) in a short time very considerably reduced its virulence, so that such an attenuated culture might be used for the immunization of animals. The same result could be obtained, as was found by Chamberland and Roux, by cultivation of the *B. anthracis* in a medium containing a small amount of an antiseptic, such as carbolic acid. Another method of reducing an organism's virulence is by passage through a series of animals, the bacteria being recovered from the body of one after its death and being injected into another. Although this procedure generally increases the virulence of the bacterium for the species used in the passage, it may lower it for other species. The method now most generally employed, especially in the earlier stages of immunization, is the injection of suspensions of dead bacteria. These may be killed either by the addition of an antiseptic such as formalin, or by heating, a temperature of  $55^{\circ}$  C. for one hour being sufficient to kill most non-sporing bacteria. After several injections of dead bacteria, the process of immunization may be continued, using living bacteria of lowered virulence, and then passing on to those of normal or exalted virulence. It may be necessary to produce immunity in animals, not against a bacterium but against some of its products, particularly its toxin. This method is successful chiefly in the case of those organisms producing an exotoxin, such as the diphtheria bacillus. The earliest injections are either small in amount or consist of toxin which has had its poisonous properties lessened. As a result of repeated injections of increasing doses of the toxin, the animal's serum becomes antitoxic—that is, it neutralizes the toxin.

If the serum of an animal, which has been successfully immunized against either a bacterium or a toxin, be injected into another, the latter becomes, to some extent, immune against the substance used. This is passive immunity. It is most successful in the treatment of diseases due chiefly to



bacterial toxins. The patient suffering from diphtheria who is given an injection of diphtheria antitoxin is passively immunized by the serum of a horse which was actively immunized by injections of diphtheria toxin. The great advantage of passive immunity over active is that there is no delay to allow of the development of anti-substances. These are present, ready formed, in the serum injected, and are able to grapple immediately with the bacteria or the toxin causing the disease. The effects of passive immunity are, however, only short-lived, as the active constituents of the injected serum are gradually eliminated and have completely disappeared in about a month, and the body cells have not been trained to produce more. In active immunity, on the other hand, the effects persist for a long time, even to some extent for life. Passive immunity is most useful in the treatment of acute diseases, active immunity for prophylaxis and for the treatment of subacute and chronic diseases.

It is occasionally possible to combine, to some extent, active and passive immunity. If a child, exposed to infection from a case of measles, is at once inoculated with a few cubic centimetres of serum of a measles convalescent he will escape the disease entirely. This is passive immunity and it will only last for a few weeks. If the amount of serum injected be small or if its administration be delayed until the sixth or eighth day after exposure, he will have a very mild attack of measles, probably unrecognizable as such and only lasting one or two days. This will be sufficient to produce an active immunity which will probably be permanent. The convalescent serum modifies the disease, but the virus of the disease develops sufficiently to lead to the production of active immunity.

## CHAPTER XIV

### THEORIES OF IMMUNITY

WE have dealt with the methods used to produce immunity, and we now must consider what differences are to be detected between an immune and a non-immune animal, and how these differences are to be explained.

Immunity may be due either to the cells or to the fluids of the body, and for several years there was acute controversy between the upholders of rival theories, the cellular and the humoral. Metchnikoff, the leader of the cellular school, believed that immunity was entirely due to certain cells, the phagocytes, found in the blood and tissues, which had the power of ingesting and destroying bacteria. His opponents saw in Metchnikoff's phagocytes mere scavengers which removed the bodies of bacteria slaughtered by various substances in the plasma of the blood. It is now generally conceded that the truth lies midway between the views of the extremists. The phagocytes are probably the most important of the body's defenders, but without the help of the plasma they are powerless. The plasma, independent of these cells, is capable of destroying bacteria and of rendering their products harmless. Both phagocytes and plasma work together in harmony with the object of ridding the body of all its microbial invaders. In the development of immunity the phagocytes do not appear to be altered or to acquire any new powers, but they do become more useful, thanks to changes in the plasma. The latter, as a result of immunization, does undergo certain changes with the development of new functions and the increase of others, formerly weak. It is, therefore, with these changes in the plasma and serum of the blood that immunity is largely concerned.

Immunity, as developed against bacteria, is but one expression of a general law that a living body strives to render harmless any foreign material introduced into it. In the case of many materials, the method adopted is by the production of a specific substance which is capable either of uniting with and rendering harmless the material introduced or of digesting it. The substance introduced is called an Antigen, and the developed substance which opposes it an Antibody.

Until a few years ago it was believed that all antigens were proteins, but it has been shown that various chemical compounds of high molecular weight, and particularly polysaccharides, can act as antigens. Certain other polysaccharides and lipoids behave as partial antigens or haptens.

The difference between an antigen and a hapten is that the former is capable both of stimulating the formation of an antibody and, if brought into contact with an already formed antibody, of interacting with it, while a hapten can interact with an antibody but, alone, cannot stimulate its production.

A natural question would be, how then is an antibody to a hapten produced? The answer is that a hapten can be converted into an antigen by combining chemically with another molecule, usually of a protein but possibly, in some cases, of a much simpler atomic group. The molecule so formed stimulates the production of an antibody which interacts with the hapten whether this be alone or combined.

When an antigen is introduced into the blood stream, directly or indirectly, there is a latent period of several days and then antibody begins to be formed. This increases in amount and, in about seven days, is at its maximum, after which it diminishes. If, however, a fresh supply of antigen be administered, the antibody concentration in the blood again increases and so, by successive inoculations of antigen, the antibody concentration can be increased to the maximum possible for the animal.

It is probable that antibodies are produced by the cells of the reticulo-endothelial system, a system wide-spread

throughout the body. We know nothing of the mechanism of their production but, whatever it may be, it is certain that the antigen molecules play an important part in arranging their structure. The evidence at present available suggests that there must be some sort of correspondence between the atomic arrangement in the molecules of an antigen and its antibody. This permits the two to combine together in the highly specific manner to be referred to later.

It may be taken as established that antibodies are globulins which, in some way not yet understood, have acquired a character in conformity with the antigen which called them into being. They are stable substances which, kept under suitable conditions, remain practically unchanged for years. They are not destroyed at a temperature of 55° C., but at higher temperatures, 65 to 70° C., they are denatured and lose their power.

When antigen is no longer introduced into the body, the amount of antibody in the blood gradually decreases. Ultimately, possibly in a year or two, no antibody may be detected, but this does not mean that the actively immunized individual has ceased to be immune. The cells which were once educated to produce a particular antibody may cease to do so when the stimulus of the presence of antigen is withdrawn, but the lesson once learned is never forgotten. The cells remain reactive: they are, so to speak, on a "hair-trigger" and, in response to the slightest stimulus, such as the introduction of a very small amount of the antigen, again produce the corresponding antibody much more rapidly and in larger amount than can the untrained cells of a non-immune animal.

A very great variety of substances can act as antigens. These substances may be actually or potentially harmful, as bacteria, bacterial toxins and snake venoms are, or they may be entirely harmless, as are the serum or cells of an animal of a different species, egg albumin or milk. The production of antibodies is not merely a method of protecting the body against bacterial disease, but a manifestation of a much more general phenomenon which appears to have, as its aim,

the removal from contact with the cells of the body of organic substances not normally present in the tissues.

The most important and characteristic feature of antibodies is the high degree of specificity which they possess for their antigens. A particular protein may be an antigen and give rise to a specific antibody. If the protein molecule be altered by the addition of a new molecular group, such as acetyl ( $\text{CH}_3\text{CO}$ ) or butyryl ( $\text{CH}_3\text{CH}_2\text{CH}_2\text{CO}$ ), each of the new compounds so formed will be antigens and each will stimulate the production of an antibody specific for itself and incapable of reacting with either the other new compound or the parent molecule.

A very limited knowledge of biochemistry suffices to show that natural products, such as blood cells, are not pure chemical substances but are composed of several of these, each of which may act as an antigen. The result, therefore, of the introduction into the tissues of an animal (*e.g.* rabbit) of blood cells of an animal of a different species (*e.g.* horse) is the production of several antibodies each specific for one of the antigens of the cells. When we mix the serum of the immunized animal with a suspension of the cells, the antibodies combine, each with its corresponding antigen, and the effect is the same as if a single antibody combined with a single antigen. If we mix this serum with the blood cells of an animal of a species distinct from, but related to, the species from which the cells used to produce immunity came (*e.g.* ass), we are able to demonstrate a combination of antibody and antigen. It would appear as if the high degree of specificity which we mentioned above did not operate in this case. But the specificity is not for the cell as a whole, but for its individual chemical components. Horse blood cells are not our antigen, but various chemical substances present in these cells. Ass blood cells also contain several substances, and some of these are identical with those in horse cells. The antibodies developed against these will react with their antigens whether in the cells of the horse or the ass. But horse cells contain some substances which ass cells do not. It is possible to remove from the serum antibodies to the antigens which are

common to both types of cell and leave behind antibodies which interact only with the antigens peculiar to the cells of the horse. The serum so treated then behaves as if it contained only antibodies specific for horse cells. The relationship between antigen and antibody is always highly specific. If there is an apparent lack of specificity, it is because the immunizing material is complex and contains more than one antigenic substance.

We are concerned with immunity chiefly as a means of defence of the body against bacteria and their products. When a man or an animal becomes immune to a bacterium, his serum contains not an antibody to this bacterium but several antibodies, each corresponding to a particular antigen in the bacterium. Such a serum may interact not only with the bacterium used to immunize, but also with other closely related bacteria: this is because they possess common antigens.

When bacteria are brought into contact with the serum of an animal immunized against them, union of antibodies in the serum with corresponding antigens in the bacteria first occurs. Then there may follow a series of events which depend on the position in the bacteria which the antigens occupy and on the environment in which the two are present. There is a considerable amount of evidence that only the more superficially placed antigens interact with antibodies. Even if the serum contains antibodies to antigens in the interior of the bacteria, their effects are masked by the surface antigens, if these are still present and the bacteria are intact. So, if a motile bacillus is used as an immunizing agent, the most obvious phenomena are those resulting from the union of the antigens of the flagella with their antibodies, or if capsulated bacteria are employed, the interaction of capsular antigens and their antibodies lead to the most easily demonstrated effects.

When bacteria are brought into contact with specific antiserum in the presence of an electrolyte, the bacteria adhere together in clumps or, in other words, are agglutinated.

When bacteria are dissolved and the solution mixed with

antiserum, the antibody, which is composed of globulin, combines with the antigen and the combined molecule constitutes an insoluble substance which is precipitated.

When an antibody combines with its antigen in intact bacteria, the latter become susceptible to phagocytosis. This is due to the opsonic effect of the antibody.

When living bacteria and their antibodies are present with complement, an unstable component of fresh normal serum, the bacteria may be killed (bactericidal effect) or even dissolved (bacteriolytic effect).

It is often convenient to refer to antibodies by names which denote the kind of phenomena which follow their union with their antigens. So we speak of agglutinin, precipitin, opsonin, bactericidin, and bacteriolysin. The antigens taking part in some of the phenomena may receive similar terms, *e.g.* agglutino-gen, precipitino-gen. Convenient as these usages are, they must not lead us to regard the antibody responsible for agglutination as distinct from those responsible for precipitation, opsonization or bacteriolysis, except in so far as the position of the antigens in the bodies of the bacteria acts as a limiting factor. It is fairly obvious that antibodies to flagellar antigens can cause agglutination but are incapable of dissolving the bacteria themselves. For this reason, they are agglutinins but not bacteriolysins.

The essential phenomenon is the union of antigen in the bacteria with antibody in the serum. The obvious features of the reaction, such as agglutination, dissolution or phagocytosis are due to secondary activities which are brought about by non-specific agencies, such as an electrolyte, complement or leucocytes.

A useful distinction is sometimes made between antibodies which can react with antigen alone (agglutinin, for example), and those which require complement to effect some change (bacteriolysin): the latter are called immune bodies. But the same antibody which, without complement, causes agglutination, may, if it reacts with a sufficiently important component of the bacterial body and if complement be present, cause the bacteria to be dissolved.

It seems to be a universal rule that, if antigen and antibody unite, complement, if present, is fixed even if it plays no obvious part in the subsequent events.

Antibodies are developed not only against the various antigens in the bodies of bacteria, but also against their soluble products such as toxin. The antibody to a toxin is called an antitoxin. It unites with the toxin and, if the two are present in correct proportion, an insoluble toxin-antitoxin molecule is formed and is precipitated. Complement is not required for the neutralization of toxin by antitoxin but, if present, is fixed.

While antibody formation is a highly important means of defence against bacteria, it is not necessarily the only one. Certain tissues of the body are stated to have the power of becoming immune without the appearance of antibodies in the circulation. This is the basis of Besredka's work on the oral administration of vaccines as a prophylactic against intestinal disease. By giving bile, followed by killed cultures, by the mouth, Besredka claims to produce a local immunity of the intestine against *Bact. typhosum*. A certain amount of agglutinin, particularly of the O type, may appear in the circulation, but this Besredka regards as only accidental. For him, the immunity is one of the cells of the intestinal mucous membrane, as is that due to the natural disease. Besredka claims that other tissues, notably the skin, can also be immunized. By intradermal inoculation of anthrax vaccine into rabbits these animals can be rendered immune. Immunity can be produced against staphylococci, according to the same authority, by intradermal administration of vaccines or by "antivirus" applied to the skin (see p. 80).

The exact position in immunity of certain curious phenomena, first observed by Twort and fully investigated by d'Herelle, has not yet been determined. d'Herelle observed that a filtrate of the stools of a convalescent dysentery patient had the power of clearing a broth culture of Shiga's bacillus. When a culture so dissolved was filtered, and a few drops added to a fresh culture, the bacilli were similarly dissolved, and this could be repeated indefinitely, more than a thousand



passages having been observed. The amount of filtrate necessary to effect clearing is very small, as little as 0.00001 c.c. being sufficient.

In addition to the characteristic clearing of broth cultures, another feature of the activity of filtrates is seen when a broth culture, after the addition of the activating agent, is spread on agar. After incubation, the uniform film of growth is interrupted by the occurrence, here and there, of circular bare areas, which may measure up to 3 mms. in diameter. In these the planted bacteria have been dissolved and no growth has taken



FIG. 35.—BARE SPACES IN CULTURE OF BACT. SHIGÆ  
DUE TO ACTION OF BACTERIOPHAGE ( $\times \frac{1}{2}$ ).

place. Only living bacteria are dissolved, dead organisms being unaffected. The active agent has not the specificity at first thought, since one filtrate may be active against not only Bact. shigæ but also against many other organisms, chiefly those of the coli-typhoid group. Further, the agent may be obtained from the fæces of patients suffering from typhoid fever or other intestinal diseases, from healthy persons, the fæces of animals, soil, water and other sources.

d'Herelle believes the active agent to be a minute, living organism, a parasite on bacteria, and his view has now been

adopted by many others. There is no doubt that the immediate cause of the dissolution of the bacteria is the action of an enzyme. The enzyme is not, however, one which came from the body of the patient, for if this were the case its activity would cease after a very few passages owing to its dilution. The enzyme is constantly being regenerated, and d'Herelle asserts that it is produced by the minute parasite to which he has given the name of Bacteriophage. The bacteriophage principle, whatever it may be, is moderately stable. It can be kept for several years and still maintain its activity. It withstands drying, exposure to a temperature of  $70^{\circ}$  C. for thirty minutes and the action of glycerol, phenol and mercurial disinfectants in strengths lethal to the majority of non-sporing bacteria. Despite claims of good results both in the treatment and prophylaxis of cholera and many other diseases, it is doubtful whether the bacteriophage plays any great part in the immunity of animals against disease. One reason for this opinion is that bacteria exposed to the action of the bacteriophage develop resistant strains, which are unaffected by it, and another is that the action of the bacteriophage is, to a considerable extent, inhibited by the presence of body fluids to the colloidal particles of which it is apparently adsorbed.

## CHAPTER XV

### PHAGOCYTOSIS

ONE of the great landmarks in the study of immunity was Metchnikoff's discovery of phagocytosis. In the body of man, as also of the higher animals, certain cells are found capable of ingesting bacteria, which have invaded the body, and of destroying them. Metchnikoff described two types of phagocytes—the microphages and the macrophages. The former of these are wandering cells, normally found chiefly in the blood stream and better known under the name of polymorphonuclear leucocytes. The latter are fixed tissue cells, and include the mononuclear cells of endothelium as well as those of connective and lymphoid tissues. In most bacterial diseases the microphages play the most important part, while the macrophages act largely as scavengers, disposing of dead cells and other waste materials. They attack certain bacteria, however, the most important of which are tubercle bacilli.

In the contest between cells and bacteria three phases are to be observed : (1) the approach, (2) the ingestion, and (3) the digestion. When bacteria are in the proximity of microphages they may attract the latter to themselves—Positive chemotaxis. It is commonly believed that they are also able to repel microphages—Negative chemotaxis—but this is less certain. The apparent repulsion may be due to a lack of attraction combined with the lethal action of bacterial products on the cells. In the early stages of a very acute infection, such as that produced by a virulent streptococcus, the bacteria may be found in large numbers with relatively few pus cells. Later, positive chemotaxis occurs, polymorphonuclear leucocytes accumulate at the site of infection and approach the bacteria. These cells are the body's chief defenders and their collection is of

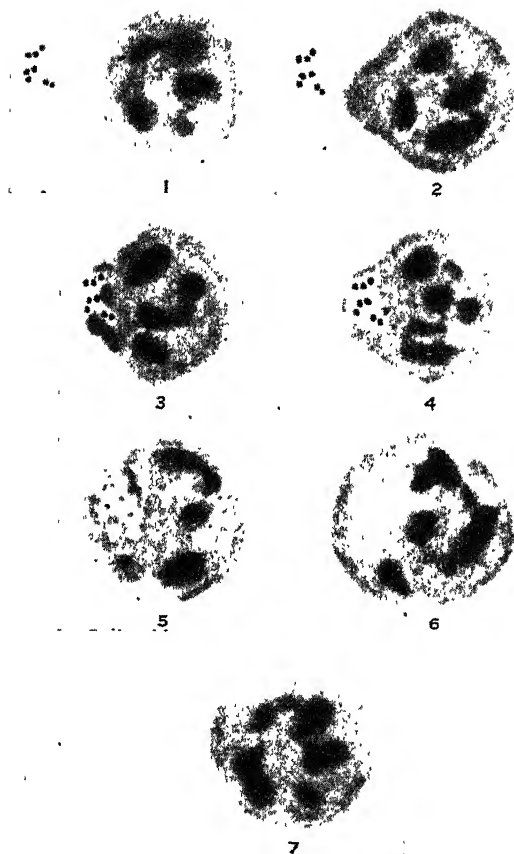


FIG. 36.—PHAGOCYTOSIS.

Diagram showing seven stages in the phagocytosis of cocci by a polymorphonuclear leucocyte.

importance in prognosis, as was recognized empirically by our ancestors in the days when almost every wound was infected, for to them "laudable pus" was a sign that all was going well, whereas a thin watery discharge was a danger signal.

The second stage is the ingestion of the bacteria which is

effected by amoeboid movements of the cells. A single cell is capable of ingesting a very large number of bacteria and may seem to be almost completely filled with them, except for the nucleus, which is not encroached upon.

The third stage (that of digestion) is carried out by ferments produced by the cells. It is probable that the number of these ferments is not large, as they do not seem to be specific for different types of bacteria. The latter, however, vary considerably in their resistance against digestion: some are dissolved rapidly while others remain, apparently uninjured, for a considerable time, and may even grow in artificial culture.

Metchnikoff did not realize that the phagocytes alone are almost entirely incapable of ingesting bacteria. If blood be taken and the cells, after washing in several changes of saline to free them from serum, be mixed with bacteria, phagocytosis does not occur. If, to the mixture of washed cells and bacteria serum be added, the bacteria are ingested and dissolved. At first sight it would appear that the serum acts on the cells, but in reality the action is on the bacteria, as can be proved by allowing the serum to act on the bacteria for some time, after which they are washed in saline to free them from all traces of serum. On mixing these treated and washed bacteria with washed leucocytes, phagocytosis occurs. The substance in the serum which makes the unpalatable bacteria into a tasty meal for the cells is called Opsonin or Bacteriotropin.

The terms Opsonin or Normal Opsonin are applied to the opsonic substances in the body of a non-immunized animal. Immune Opsonin or Bacteriotropin is an antibody developed against bacterial antigen. It is found only in the immunized animal. There are certain differences in the resistance of the two to heat, bacteriotropins being more thermostable, but their results are the same—alteration in the bacteria whereby they become capable of being phagocytosed. It is probable that, in both, similar antibodies are concerned but that, in the case of opsonins, complement is essential for their action, while in the case of tropins complement may assist but, in its absence, the tropins are still active. A

further difference is that normal opsonins are non-specific being capable of acting on several varieties of bacteria and, if absorbed by one type of bacterium, all opsonic activity of the serum is lost. Tropins, on the other hand, are specific. If they were developed in response to the stimulus of a staphylococcus they will act only on that organism and not on, say, tubercle bacilli. Tropins, when present, are much more active, that is, they act in much higher dilution than do normal opsonins.

The opsonic index, which is a measure of the opsonic power of a serum, varies during the course of a disease. When below normal (taken as 1) the patient's resistance against the test organism is lowered. When the disease has been cured, the index is found to be raised; that is, the immunity of the individual is greater than that of a normal person. Wright found that the index could be raised and immunity produced by the administration of vaccines, the result being the development of tropins specific for the bacteria employed.

The first result of the injection of a vaccine is to lower the opsonic index (negative phase). This is followed by a rise of the index to a higher level (positive phase). The danger of lowering immunity by administration of a vaccine is slight if the injections are given at a sufficient interval (four days or more). Each injection causes some lowering of the index; but this is followed by a rise to a point higher than previously so the net result is a rise in the index and an increase in the immunity against the bacterium used.

## CHAPTER XVI

### TOXINS AND ANTITOXINS

ONLY a limited number of bacteria produce exotoxins to any considerable extent. The most important of these are *C. diphtheriæ*, *Cl. tetani*, *Str. pyogenes*, *Staphylococcus pyogenes*, the Shiga dysentery bacillus and *Cl. botulinum*. Of these the toxin of *C. diphtheriæ* was first investigated, and against it an antitoxic serum was first prepared by v. Behring. It may, therefore, be taken as a type of exotoxin, and the relation of it to its antitoxin may be discussed at some length.

In the first place, it should be realized that although we speak, rather incorrectly, of a filtrate of a broth culture as toxin, the actual pure toxin has never been isolated. We know, however, that the toxins are nitrogenous substances, probably allied in chemical composition to the albumoses. They are soluble and non-crystallizable. They are slightly dialysable, that is, they can pass through only a somewhat permeable membrane. They are rather unstable, being destroyed by heat, light, exposure to oxygen and various chemical substances.

The chief characteristic which distinguishes toxins from poisons of known composition is that the repeated administration of sub-lethal doses of toxin leads to the animal becoming immune, so that ultimately it is unaffected by an amount representing possibly several hundred times the usual lethal dose. This immunity is due to the presence, in the animal's serum, of some substance, called antitoxin, which deprives the toxic filtrate of its poisonous properties. The earliest theory of the formation of antitoxin regarded it as a toxin which had been modified in the animal's body. That this is untenable is shown by the fact that the amount of

antitoxin produced may be many times that of the toxin injected. The antitoxin, then, is developed as a response to the stimulus of the injected toxin; but is it an entirely new substance produced by the body, or merely an enormous increase in some substance previously present in small amounts? For several reasons, the latter view is more likely to be correct. Most animals normally possess, in their sera, small amounts of antitoxin.

It is obvious that, in order to inflict harm on the living animal, toxin must enter into some form of combination with susceptible cells in the animal's body. Support is given to this belief by the fact that a mixture of a toxin with certain cells (tetanus toxin and nerve cells), after an interval has elapsed, is found to be non-toxic on injection into an animal. Again, if tetanus toxin be injected into the blood stream, it disappears very quickly, although no symptoms appear for about eight hours. If the animal which has received an injection of tetanus toxin be bled completely at once, the blood being replaced by that of a normal animal, symptoms will come on exactly as if it had not been bled. The toxin must have been rapidly removed from the circulation and fixed somewhere in the body—most likely to vulnerable cells. Antitoxin acts by combining with toxin and so preventing the latter from entering into combination with cells.

An animal may be insensitive to the action of toxin for two reasons: its cells may not be capable of combining with toxin or, although such combination may occur, the toxin inflicts no damage on the cells. In neither case would the animal be injured, but, in the first, toxin injected would remain free and uncombined in the circulation and, in the second, the toxin, being cell-bound, would disappear and antitoxin might be produced. Such cases are actually known. Diphtheria toxin is almost non-toxic for the rat, and this animal may have in its circulation, twenty-four hours after toxin has been injected, sufficient free toxin to kill a guinea-pig. The alligator is insensitive to tetanus toxin but, after an injection, the toxin disappears from the circulation. If the animal be kept at 20° C., no antitoxin is produced, but



by keeping it at a higher temperature ( $30^{\circ}$  to  $37^{\circ}$  C.) antitoxin is formed. In the rat no combination between toxin and cells is effected; in the alligator, union takes place. The toxin is non-toxic for the alligator, but is antigenic and an antibody (antitoxin) is produced.

Toxin and antitoxin combine outside the body and the toxin is neutralized but this union does not occur instantaneously. The molecule of toxin is able to pass through a porcelain filter, the pores of which have been blocked with gelatin, but antitoxin is unable to pass. If mixtures of toxin and antitoxin are made and filtered at various periods after mixing, it is found that the amount of toxin passing through is less the longer the time elapsing between mixing and filtration. If filtration is carried out immediately the two substances are mixed, all the toxin passes through, but if there is a delay of two hours no toxin is found in the filtrate.

That antitoxin merely combines with toxin, and so prevents its action without injuring it, is shown by the fact that the toxin-antitoxin combination can be dissociated. Different batches of antitoxic sera vary in the avidity with which the antitoxin combines with toxin. With some the union is so very loose that estimations of the strength of antitoxin are difficult. An example of the dissociation of toxin and antitoxin was furnished by some deaths which occurred among children inoculated with a diphtheria toxin-antitoxin mixture. This had originally contained no free toxin but, as a result of freezing, there had been a local concentration of phenol which had destroyed the antitoxin, leaving toxin free. Many other similar methods of dissociating toxin-antitoxin combinations have been demonstrated.

Antitoxin is usually obtained from horses. A potent toxin, the method of preparation of which differs in the case of different bacteria, is the first requisite. Toxins are powerful poisons and their injection may kill or inflict serious injury on the animals into which they are injected. Fortunately, the majority of toxins can be converted into toxoids by the action of formalin. A toxoid is non-poisonous but behaves antigenically in exactly the same way as the toxin from which

it was derived. The antibody developed against a toxoid is identical with that developed against the corresponding toxin, that is, it is antitoxin. Injections of increasing doses of toxoid are given at intervals. These are usually administered subcutaneously. Finally toxin is substituted for toxoid. After a course lasting one or two months, the horse may be able to withstand an injection of several hundred cubic centimetres of active filtrate. When its serum contains a satisfactory amount of antitoxin the animal is bled. It is not bled to death as, after an interval and a further course of injections, it may be bled again. With proper care, a horse may continue to give useful antitoxic serum for a year or more. The blood is allowed to clot and the serum, freed from cells, is bottled for use after the addition of a small amount of phenol or other antiseptic.

Like other antibodies, antitoxins are intimately connected with the globulin fraction of the serum. Based on this fact are various methods of concentrating antitoxin, such as by the addition of ammonium sulphate to the serum, which has the effect of precipitating antitoxin together with globulin. The precipitate is purified and re-dissolved. In this way a greater unitage of antitoxin can be obtained in a given volume than in the case of raw serum. This concentrated antitoxin has the further advantage that it is less likely to cause serum sickness than is pure serum.

Since the concentration of antitoxin in the sera of different animals, immunized against the same toxin, differs considerably, it would be very unsatisfactory to describe a therapeutic dose in terms of the volume of serum administered. We must, therefore, have, for each antitoxin, a unit in terms of which the amount of antitoxin in a given volume of serum may be described.

The first such unit was that propounded by Ehrlich for diphtheria antitoxin. Ehrlich commenced by defining the unit of toxin, which he called the minimum lethal dose (M.L.D.), as the amount of toxin which killed a guinea-pig weighing 250 gms. in four days. His unit of antitoxin was that amount which, mixed with 100 M.L.D. of toxin, pre-

vented the death of a guinea-pig of standard weight within four days or, in other words, the amount which almost neutralized 100 M.L.D. of toxin.

It will be realized that Ehrlich, in effect, adopted as his fundamental unit a certain dose of toxin. Experience soon showed that this method was unsatisfactory. Toxin is an unstable substance: as it ages, larger doses of the toxic filtrate are required to kill guinea-pigs or, in other words, the M.L.D. increases. This is due to a conversion of toxin into toxoid which is non-lethal. Measurements such as the  $L_+$  dose, to be described later, which estimate toxin plus toxoid in terms of power of combination with antitoxin, remain practically constant as the filtrate ages.

The unit of every antitoxin, including that of diphtheria, is now defined as the amount of antitoxin in a certain volume of a particular serum or, to be more exact, in a certain weight of a particular dried serum, for the standard antitoxins are kept dry and in the cold, in which condition their potency remains unaltered for years.

Ehrlich's original unit of antitoxin, derived as explained above, was adopted as the internationally recognized standard unit and new antitoxic sera are standardized, directly or indirectly, against it, but it is regarded merely as an arbitrary unit. The position is similar to that of the metric unit of length, the metre. This was originally selected as being one ten millionth of the distance between the north pole and the equator and a bar of metal was constructed which represented this length. The official standard unit is now this bar, and no one would think of making fresh standards according to the method originally used.

The titration of a new diphtheria antitoxic serum may be described in some detail as the principles on which the method is based are substantially the same as those underlying the titration of any other antitoxic serum.

A weighed amount of dry standard antitoxic serum, obtained from one of the research institutes in which standard sera are preserved, is dissolved in water so as to give a solution containing exactly 1 unit of antitoxin in a given volume.

This volume is measured into a series of tubes, to which are added decreasing volumes of a toxic broth filtrate. After the mixtures have stood for some time, each is injected into a guinea-pig of standard weight. Some of these animals die rapidly, others slowly. The one which dies in four days is considered to have received an  $L_+$  dose of toxin, for the  $L_+$  dose is defined as that amount of toxin which, when mixed with 1 unit of antitoxin, kills a guinea-pig in four days. Then another series of tubes is taken and to each is added the amount of the toxic filtrate which contains 1  $L_+$  dose of toxin. Decreasing amounts of the new antitoxic serum are added to these and, as before, the mixtures are injected into guinea-pigs. Since 1  $L_+$  dose of toxin plus 1 unit of antitoxin kills a guinea-pig in four days, and since each animal received 1  $L_+$  dose of toxin, the amount of the new serum in the mixture injected into the guinea-pig which dies on the fourth day must contain 1 unit of antitoxin.

Many other antitoxic sera are standardized in a similar way, the guinea-pig being used for some, the mouse for others. In every case, one unit of antitoxin is present in that amount of a new serum which, plus a certain volume of a toxic filtrate, produces exactly the same effect (*e.g.* death in a given time) in the experimental animals as the same amount of filtrate plus one unit of standard antitoxin.

An alternative method of titrating diphtheria antitoxic serum is commonly employed. This is the intradermal injection of mixtures of toxin and antitoxin. The subsidiary unit of toxin used is the  $L_r$  dose. This is the amount which, injected intradermally into a guinea-pig together with one unit of antitoxin, causes a reaction in the form of a red flush 5 mms. in diameter within thirty-six hours. First, using a standard antitoxin, the  $L_r$  dose of a toxin is determined. Then, using the same dose of toxin, the amount of the new serum which permits just the same degree of damage to occur is ascertained: this contains one unit of antitoxin. This intradermal method has the advantage of cheapness, since a number of tests may be made in the same animal, and the further advantage that, by using the same animal for several

tests, the errors introduced by the different susceptibilities of different animals to diphtheria toxin are eliminated.

For standardizing the antitoxin to the erythrogenic toxin of *Str. pyogenes*, the skin of a susceptible human being is usually employed, although the rabbit and goat are sometimes used.

The antitoxins to toxins which are also hæmolysins (such as the  $\alpha$  and  $\beta$  toxins of *Staphylococcus pyogenes*) may be standardized by finding the amount of the standard and new sera which neutralize the lytic effect on red blood cells of the same volume ( $L_h$  dose) of the toxin.

Another *in vitro* test, that of Ramon, is also employed. This depends on the fact that when, to a series of tubes, a constant amount of serum and decreasing amounts of filtrate are added, flocculation occurs, appearing first in one tube and then spreading to adjacent tubes in both directions. The tube in which it first appears contains a neutral mixture. The  $L_t$  dose of a toxin is the volume which, when mixed with one unit of a chosen antitoxin, flocculates more rapidly than does any other volume with the same amount of the same antitoxic serum. This method is commonly employed in preliminary estimations of the strength of a new antitoxic serum. In using this method it should be remembered that the neutral point is the one at which both toxin and toxoid are neutralized by antitoxin. The test shows the total combining capacity of the filtrate for antitoxin, but not the amount of toxin present. The  $L_t$  dose of a toxic filtrate is not significantly altered when toxin is changed to toxoid. The antigenic value of a preparation is frequently stated in  $L$  units, that is the number of  $L_t$  doses in each cubic centimetre. Active immunity is best obtained when a preparation with a high value in  $L_t$  units is used.

In most civilized countries, legislation exists to enforce the labelling of serum containers with a statement of the strength of the serum in recognized units. So, in the British Islands, the unit of diphtheria or tetanus antitoxin has just as good a legal status as has the yard or the pound. Under the auspices of the League of Nations, international agreement

has been reached concerning the chief antitoxins and a unit of one of these is the same in London, New York, Dublin, Paris, and Copenhagen.

Ehrlich believed that toxin combined with antitoxin as does a strong acid with a base. Arrhenius and Madsen suggested that the two reacted as do a weak acid, such as boric acid, which is not completely dissociated, and a base like ammonia. In a mixture of these substances there are always present boric acid, ammonia and ammonium borate. The relative proportions of the three depend on the concentrations of the acid and base. In a neutral toxin-antitoxin mixture there are always present free toxin and antitoxin, as well as toxin and antitoxin combined. This theory seems to agree better with the facts than does that of Ehrlich. Bordet's view is that the action of antitoxin on toxin is not a chemical one, but resembles the physical phenomenon of adsorption. Just as a stain is adsorbed on filter paper, so the relatively small molecule of toxin is adsorbed on the larger molecule of antitoxin. A fourth theory is that toxin and antitoxin combine in definite proportion and that the compound formed can adsorb either toxin or antitoxin according as one or other remains free after the combination has been effected.

## CHAPTER XVII

### AGGLUTINATION

IF a broth culture of *Bact. typhosum* be mixed with the serum of a patient taken during, or soon after, an attack of typhoid fever and observed under the microscope, two things are seen to happen: first the bacilli lose their motility, and secondly they adhere together in clumps. This second phenomenon is known as Agglutination. In many bacterial diseases as well as in typhoid fever, the patient's serum acquires this property of agglutinating the causative organism. It is not essential that the organism should be motile, since the phenomenon occurs with non-motile or even dead bacteria.

Agglutination can best be studied, not with the sera of human patients, but with those of animals which have been immunized with dead or living bacteria. Agglutination of bacteria by a specific serum may be observed microscopically or macroscopically, the latter being the better method. The titre of a serum is the highest dilution at which it produces agglutination. It depends to some extent on the duration and temperature of incubation, on the strain of bacteria used and on the strength of the suspension. It is not difficult to produce a serum with a titre of one in several thousands against members of the typhoid-colon group of bacilli; but against other bacteria a much lower titre may be regarded as satisfactory.

As a matter of convenience, when referring to agglutination, we may speak of the antigen in the bacteria as agglutino-gen and the antibody in the serum as agglutinin. This does not imply a belief that the antibody which we call agglutinin can only cause agglutination. If its antigen is suitably situated in the bacterial body, it may act as opsonin, bactericidin or bacteriolysin.

In agglutination there are two phases: first, the union of the agglutinin with the agglutigen, and secondly the clumping. That the two phenomena are distinct was shown by Bordet. Agglutinating serum and a suspension of bacteria were freed from salts by dialysis and mixed together; no agglutination occurred; but if a trace of sodium chloride or other electrolyte was added the bacteria formed clumps. That a definite combination of agglutinin with agglutigen took place was proved by thoroughly washing the bacteria, after they had been in contact with the serum for some time, in several changes of distilled water, centrifuging each time to collect the bacteria. On adding salt to the suspension of these washed bacteria in water agglutination occurred almost immediately. The union of agglutinin and agglutigen is specific, and is probably chemical in nature. As a result of the union some alteration, probably physical, is produced in the bacteria which permits them to be agglutinated in the presence of electrolytes. The actual agglutination is a purely physical phenomenon which is largely non-specific. Agglutinins do not require the presence of complement for their activity.

Owing to the facility with which agglutination can be studied, the amount of agglutinin in a serum is often estimated in order to assist diagnosis or to measure immunity. While the former, properly controlled, is legitimate, the latter may be fallacious if the floccular type of agglutination is considered. There is reason to believe that the O type of agglutination may supply a means of determining an individual's immunity to an organism, for the O antigen appears to constitute the toxic factor in bacteria.

It is worthy of note that an organism, normally motile, when freshly isolated from the body may be non-motile owing to a temporary loss of flagella and so be inagglutinable as judged by the floccular (H) type of agglutination and that bacteria grown in agglutinating serum may behave in the same way. After a few generations on artificial medium the motility and agglutinability in either case returns.

It has been said above that an agglutinin is specific against



an agglutinin which calls it forth. It is necessary to point out, however, that the agglutinin is not the bacterial body as a whole but the separate molecules which go to the composition of this body. The specificity, in fact, is to be regarded as chemical rather than biological. Bearing this in mind, the apparent lack of specificity of some agglutinating sera is not hard to understand. An animal is immunized against *Bact. paratyphosum* B and its serum is found to agglutinate that bacillus in a dilution of 1 : 5000. It also agglutinates the fairly closely related *Bact. typhi-murium* at 1 : 2000. The explanation usually given is that the body substance of the *Bact. paratyphosum* B contains a number of definite chemical entities, each of which acts as an antigen. The body of the *Bact. typhi-murium* similarly consists of a number of chemical substances and the two bacilli have certain of these antigenic substances in common. The serum contains agglutinins against all the antigens of the paratyphoid B bacilli, and those produced against antigens common to both bacilli act on such antigens in the bodies of *Bact. typhi-murium*. The agglutinins produced against the bacterium used for immunization are called specific or major agglutinins ; those affecting other closely related bacteria are known as group or minor agglutinins. The conception that the body of a bacterium is composed of a number of distinct antigens, some of which may also occur in the body of a bacterium of a different species, is called the "antigen mosaic."

There is a close connection between the form of the colony and the behaviour of the bacteria in agglutination. Reference will be made chiefly to the intestinal bacilli since these organisms have been investigated in greater detail in this connection than have other types. A motile bacillus gives rise to the normal smooth form of colony (N). Occasionally another colony form is produced which is also smooth but which may differ slightly in other respects from the N colony. This is called the S form of colony. If agglutinating sera are prepared against the bacilli in the two types of colony it is found that serum prepared against N type bacilli agglutinates N bacilli in large loose floccules, but agglutinates

S bacilli and N bacilli, which have been heated or treated with alcohol, in small granular clumps. Serum prepared against S type bacilli agglutinates N and S bacilli, whether unheated or heated, in small granular clumps. From this it is clear that two type of agglutinin (H and O) are concerned.

N bacilli contain both H and O agglutinogens, S bacilli only O agglutinin. H agglutinin is destroyed by heat or alcohol, O is not. When N bacilli are used to immunize animals, H and O agglutinins are produced. When S bacilli or heated N bacilli are used only O agglutinin is produced.

The H type of agglutination (in large loose floccules) occurs only with motile organisms and the H agglutinogens are associated with the flagella and so are sometimes referred to as flagellar; they are heat labile. The O type occurs with non-motile organisms or with motile organisms which have been heated or treated with alcohol and the agglutinogens are called somatic or body agglutinogens; they are heat stable. The change N→S in the type of colony is due to a degradation of the bacilli with a loss of H antigen.

Since the flagella are relatively unimportant and superficially placed structures, the antibodies developed against their antigens can do little more than cause agglutination: they are almost entirely agglutinins. Antibodies developed against somatic antigens play important parts in other phenomena in addition to agglutination.

A third colony type (R) occasionally develops. It is distinguished from both N and S colonies by the fact that it is more or less rough on the surface and forms suspensions in saline which readily deposit. Bacilli in such a colony contain a third type of agglutinin (R) which resembles O in being heat stable but is, nevertheless, distinct from O. Bacilli in R colonies may be either motile or non-motile. If motile, they contain both H and R agglutinogens; if non-motile, only R agglutinin.

The S→R change is also due to a degradation, the bacilli losing their O antigen. It is probable that the R antigen is not newly acquired, but only becomes active as an antigen when the O antigen is lost. Later work suggests that R

antigen may be lost and a further antigen ( $\rho$ ), previously latent, become dominant. The various changes which a bacillus may undergo in the progression  $N \rightarrow S \rightarrow R \rightarrow \rho$  may be more clearly grasped if the bacillus is pictured as resembling an onion, various layers of which may be removed each revealing a fresh one hitherto concealed and if, as seems probable, only the antigen or antigens close to the surface stimulate the production of agglutinin. In the normal bacillus both H and O antigens are active. In the smooth variant H has been lost and O alone is active. In the rough variant O is lost and R alone is active. The last stage is the loss of R antigen leaving  $\rho$  as the active antigen. The analogy of the onion must not be pushed too far, as the loss of the various antigens by the bacillus is not accompanied by a decrease in size, as is the loss of the layers by the onion.

Complications are introduced by the fact that in ordinary cultures bacilli of N, S and R types may be present in varying proportions. In single colonies, however, the bacilli are predominantly of one type. Although a culture of an organism may have only one active type of agglutinin (O or R) there may be present a number of distinct agglutinogens all belonging to the same type. So the bacteria in a normal colony may have several H agglutinogens and several O agglutinogens; those in a smooth variant colony several O agglutinogens, and those in a rough colony several R agglutinogens. The variations from N to S and to R type of colony occur under conditions not completely understood, and it is owing to such variations that pure cultures of an organism are, now more readily and now less readily, agglutinated by the same serum.

In general, R agglutinogens are cosmopolitan, the same agglutinogens occurring in widely different genera of bacteria, while O agglutinogens are more specific, being usually confined to one genus. Several species, within the genus, may, however, have one or more O agglutinogens in common. The same O agglutinin, for example, occurs in *Bact. typhosum*, *Bact. enteritidis*, and in several other closely related bacilli.

In the case of certain members of the *Salmonella* group (*Bact. paratyphosum* B, *Bact. typhi-murium* and others which are diphasic) the H agglutinogens exhibit a curious phasic alternation between those which are specific or almost specific for the particular species (specific agglutinogens) and those which are common to various members of the group (group or non-specific agglutinogens). This finding is due to the work of Andrewes, who also discovered that a single bacillus contains either specific or group agglutinogens and not both. An ordinary culture, consisting of innumerable bacilli, will contain both, but, by special methods, it is possible to obtain cultures consisting almost exclusively of either the group or specific type. If a pure specific culture is used to immunize an animal, the serum of the latter will be specific and will be without effect on other organisms in the group. Immunization with a pure group culture produces a serum which agglutinates all the bacteria in the group indiscriminately. For the complete identification of an organism of the *Salmonella* group, among the members of which various O, H specific and H group antigens are distributed, it is necessary to state which of these antigens the organism possesses. By international agreement, O antigens are described by Roman numerals, H specific antigens by small letters and H group antigens by Arabic numerals. The antigenic composition of an organism may be very shortly described by a formula. That for the *Bact. paratyphosum* B, for example, is—IV, V, XII : b : 1, 2.

Felix has shown that highly virulent typhoid bacilli, in the living state, may be inagglutinable by serum containing O type agglutinin, owing to the presence in them of another antigen, known as Vi antigen, but that agglutination occurs, either when the same serum is used and the Vi antigen has been destroyed by some appropriate treatment or when a serum which contains an antibody to Vi antigen is employed against living bacilli. The presence of a large amount of uninjured Vi antigen in a bacillus apparently masks its O antigens, so that the organism is not agglutinated by a serum containing antibodies to the O antigens. Bacilli, which

have less Vi antigen, are agglutinated in the living state by either Anti-Vi or Anti-O sera. Up to the present, Vi antigens have been found to occur only in *Bact. typhosum* and *Bact. paratyphosum* C.

All bacteria do not behave in the same manner as the *Salmonella* bacilli. In the case of pneumococci, since these organisms are without flagella, agglutination is always of the O type. Both smooth and rough colonies are produced, the differences being that the cocci in the former have capsules, while those in the latter have not. The capsule, which consists of a carbohydrate, confers type specificity and the serum of an animal immunized with capsulated cocci agglutinates only cocci of the same type. Since the carbohydrate acts as a hapten the same serum can react with a solution of it, causing precipitation, but if the carbohydrate, without cocci, be inoculated into an animal no antibody is produced. Cocci, deprived of capsules, act as antigens, but the serum of an animal inoculated with them is species- and not type-specific, that is, it agglutinates all pneumococci whatever their type.

Agglutination may be used for diagnosis in two ways: either the serum of a patient may be tested against known bacteria or an unidentified bacterium may be tested with a known anti-serum. In neither case is it common for the presence of group agglutinins to present serious difficulties, if care is taken to carry the dilutions of the serum up to its full titre. In some cases, however, as with *Bact. paratyphosum* B and *Bact. typhi-murium* group agglutination may be so marked that it is impossible to identify the organism by the use of agglutinating serum even at full titre. It is then necessary to employ the absorption of agglutinin technique. If an antiserum is prepared for an organism and if some of it is mixed with a very dense suspension of the bacteria in question and left in contact for a few hours, all agglutinins present in the serum will become fixed to agglutinogens in the bacteria. If then the bacteria are removed by centrifuging, it will be found that the serum so treated is no longer able to agglutinate the bacteria against which it was

prepared. From this it is usually concluded that, if an unknown organism absorbs all agglutinin from a serum, so that it fails to agglutinate the homologous organism, then the unknown and homologous organisms are identical or, in other words, if an anti-P serum is treated with an unknown bacillus X and, after treatment, is found to have lost its power of agglutinating bacillus P, then X is identical with P. This conclusion is, however, not always valid. If X contains agglutinogens *a*, *b*, *c*, *d* and *e*, and P contains agglutinogens *a*, *b*, *c* and *d*, X will absorb all agglutinin from anti-P serum, but P will not absorb all agglutinin from anti-X serum. To prove the complete identity of two bacteria the "Mirror test" must be employed. Only when X absorbs all agglutinin from anti-P serum and P absorbs all agglutinin from anti-X serum are P and X absolutely identical.

Elsewhere it is pointed out that although antibodies may have disappeared from the serum, a very slight stimulus is sufficient to cause their reappearance owing to the reactive condition of the cells which produced them. A man who has been inoculated with a typhoid vaccine, and from whose serum typhoid agglutinin has subsequently disappeared, will develop a higher agglutinin titre against that bacillus in response to a further injection than one who had no previous inoculation. In the same way the agglutinin titre for the organism rises very rapidly in a person who, formerly vaccinated, develops typhoid fever. But this rise is not always specific, and difficulties in diagnosis are caused by the anamnæstic reaction which is the reappearance of antibodies in response to a non-specific stimulus. This is seen in the production of agglutination, to high titre, by the serum of a man once vaccinated with typhoid bacilli, but whose present febrile illness is due to some quite different organism. This occurs chiefly in the H type of agglutination and, by relying on O type of agglutination, errors of diagnosis from this cause are less likely to occur.

In titrating a serum it is sometimes found that the tubes containing the largest amount of serum may show little or no agglutination. This may be due either to the presence in

the serum of some protective substance—possibly a colloid—which prevents clumping but which is diluted out in lower concentrations or to a too wide departure from the optimum proportion between antigen and antibody which is so important in precipitin reactions and which cannot be ignored in agglutination. The existence of these zones of inhibition or pro-agglutinoid zones is a matter of some practical importance, as a serum may fail to agglutinate a bacterium in high concentration (*i.e.* in the earlier tubes in the series of dilutions), but agglutinates normally in the tubes in which it is more highly diluted.

Agglutinins can also be produced against other cells, such as the red cells of the blood, by injecting them into an animal of a different species. These are known as hæmagglutinins. In some cases iso-agglutinins can be produced by injecting the red blood cells of an animal into another of the same species. Iso-agglutinins occur naturally, two agglutinins and two agglutinogens being known to be present in man and, in various combinations, to be responsible for the four types of human blood, so important in connection with blood transfusion.

As has repeatedly been pointed out, an antibody to a bacterial antigen may demonstrate its presence in the serum of an immunized animal in a variety of ways when this serum is mixed with the antigen. If a clear extract of bacteria (such as that obtained by filtering through a porcelain filter a broth culture which has undergone autolysis or by dissolving a suspension of bacteria in some chemical solvent) is mixed with a specific antiserum, a precipitate appears. The soluble bacterial substance may, for convenience, be called the precipitinogen, and the antibody in the serum the precipitin. The terminology occasionally presents difficulties since, in agglutination, it is the agglutinogen, present in the bacilli, which flocculates and is deposited whereas, in precipitation, the visible precipitate comes almost entirely from the serum (which contains the precipitin) and not from the precipitinogen. Very minute traces of the extract of bacteria are sufficient, but the serum must be only moderately dilute.

For this reason, in titrating a precipitating serum, it is usual to add to each of a series of tubes a constant amount of the antiserum and decreasing amounts of the antigen. Despite their apparent differences the two phenomena are essentially similar, and may be reconciled if we remember that the union between antigen and antibody is the important thing, while the precise nature of the after events, whether agglutination of bacteria or production of a visible precipitation (consisting chiefly of globulin), are almost certainly non-specific phenomena.

In precipitation reactions the quantitative relationship of precipitin and precipitinogen is of the greatest importance. There is an optimum proportion, and in tubes containing the two reagents in that proportion precipitation occurs more rapidly than it does when they are present in any other proportion. For maximum precipitate formation, however, a greater amount of precipitinogen, relative to precipitin, is required.

In bacteriology, the precipitin reaction is used chiefly for the assignment of a streptococcus to its appropriate group by the Lancefield technique. It plays a part in typing pneumococci since, in cultures of these organisms, much of the capsular substance goes into solution and the reaction obtained with a specific serum is partly agglutination and partly precipitation. Occasionally the antigens of an organism dissolve in the body fluids and the addition of a specific serum to one of these may give rise to precipitation, so aiding diagnosis. This is true of both pneumococcal and meningococcal infections. Apart altogether from bacteriology, it has a much wider sphere, for it has been found that many substances can act as precipitinogens—the serum of various animals, muscle protein, the albumin of eggs, milk and other materials. It is extensively used in medico-legal practice in the identification of blood stains, as by its use it is possible to assert, with a considerable degree of certainty, the species of animal from which the blood in an old stain came. The blood of man, for instance, causes a precipitate to appear when mixed with an anti-human precipitating serum. No other



animal's blood, except that of some of the higher apes, gives a similar reaction. The precipitin reaction has also been used to identify the meat found in sausages and pies, in which it has been discovered that the horse has masqueraded as beef and the cat as rabbit.

## CHAPTER XVIII

### THE KILLING AND DISSOLVING OF BACTERIA AND OTHER CELLS

THE important functions of the body cells in immunity have already been mentioned, and it has been pointed out that serum is essential for their activities as destroyers of bacteria. Apart altogether from the presence of cells, serum is capable of killing certain bacteria, as was first observed by Buchner. He found that this property was present in fresh serum but was lost on keeping or on heating the serum to 60° C. To the thermolabile substance of serum, which has the property of killing bacteria, Buchner gave the name "Alexin."

To Pfeiffer is due the credit for discovering bacteriolysis. By injections of cholera vibrios he was able to immunize a guinea-pig so that it was capable of withstanding a dose of these bacteria which would have been fatal to a normal animal. He injected some of the vibrios into the peritoneal cavity of an immune guinea-pig and, from time to time, removed some of the fluid from the cavity and examined it microscopically. In this way he noted that the vibrios underwent a degenerative process. At first they lost their motility, and later they became swollen, granular, and badly staining, and finally were dissolved so that not a single vibrio could be found in the fluid of the peritoneum (Pfeiffer's phenomenon). Later, he found that if some peritoneal fluid of an immune guinea-pig were introduced into the peritoneal cavity of a normal animal along with cholera vibrios, the latter were dissolved in exactly the same way as occurred in an immune animal. The heating of immune fluid did not prevent this occurring. Bordet was able to reproduce the same phenomenon *in vitro*, using some serum or peritoneal fluid which had been previously heated to 70° C. and the fresh fluid or serum from a normal animal.

It was then clear that for bacteriolysis two substances were required, one of which was produced as the result of immunization, the other being present in fresh normal serum. The former was thermostable, being destroyed only slowly at a temperature of over 70° C.; the latter was rapidly destroyed at a temperature of 55° C. or more slowly at air temperature. Bordet called the thermostable substance the "Substance sensibilisatrice," and the thermolabile substance "Alexin," which name had previously been used by Buchner in a slightly different sense.

Following the nomenclature of the side-chain theory, a theory propounded by Ehrlich which was formerly widely held, the name of Bordet's substance sensibilisatrice was changed to "Amboceptor" and that of his alexin to "Complement." Amboceptor, according to Ehrlich, has a double linkage, uniting complement to bacteria, and hence the name given to it. Bordet's idea is that the substance sensibilisatrice sensitizes the cell and so lays it open to the destructive action of complement. It is unfortunate that active agents in immunity should receive names based on a mere theory. We prefer, therefore, to call amboceptor "Immune Body," which is descriptive without implying any theory, or, when we are considering mere killing of bacteria, Bactericidin or, if lysis is also involved, Bacteriolysin. The name complement is now so commonly used that it would be impossible to change it. French workers always call the substances by Bordet's names—substance sensibilisatrice and alexin.

Antibodies developed against antigens situated in the bodies of bacteria may give rise to either bactericidal or bacteriolytic phenomena. The two are substantially the same, the difference being due to the bacteria rather than to the antibodies. Some bacteria (staphylococci) are resistant, and so their bodies, after death, are less easily dissolved; others (vibrios) are dissolved almost immediately they are killed.

The immune body is enormously increased in amount during the process of immunization. Complement, however, cannot be increased by immunization. Complement is found

in practically every fresh serum, although its amount varies in different animals. The school of Metchnikoff holds that it is derived from the phagocytes, hence their name for it, cytase, but there is no general agreement on the point. It is rather remarkable that the amount of immune body in an immune serum is disproportionately large as compared with the amount of complement. A bactericidal serum, when complement from a normal animal is added to it, may act in a dilution of  $\frac{1}{1000}$  or more ; alone with only its own complement it is rarely active beyond  $\frac{1}{100}$ .

If in a bactericidal experiment a series of tubes be taken, each receiving a constant amount of complement and bacteria but decreasing amounts of heated immune serum, it is frequently found that the bacteria are not killed in the tubes with the largest amount of the immune serum, while in those exposed to the action of lesser amounts of this substance the organisms are killed. This, the phenomenon of Neisser and Wechsberg (deviation of complement), is comparable to the pro-agglutinoid phenomenon, and the explanation is similar to that given for the inhibition of agglutination.

In order to demonstrate the production of bactericidin by an animal, the type of bacteria used for immunization is of the greatest importance. It has been established that the antibodies produced against the bodies of bacteria (O type) are essential, H and R antibodies being practically useless for the production of solid immunity.

Lysis may be demonstrated not only with bacteria but also with other cells, particularly red blood cells. The sera of certain animals have the property of lysing the red cells of animals of a different species. Bordet found that, by injecting one animal with the blood cells of an animal of another species, the serum of the animal receiving the injection became hæmolytic for the foreign cells. For hæmolysis two substances are necessary, exactly as in the case of bacteriolysis. One is complement and the other may be called substance sensibilisatrice, immune body, amboceptor or, to give it a more specific name, Hæmolysin. Lysins may similarly be prepared against other body cells, such as those of the liver

or kidney, or against spermatozoa. These cytolysins are less specific in their action, since they are usually, to a considerable extent, hæmolytic also. The actions of various lytic sera, whether against bacteria (bacteriolysins) or against cells (cytolysins, including hæmolysins), are all identical; but on account of the ease of manipulation and the fact that lysis is most easily observed in blood cells, the action of sera prepared against red blood cells has been most studied. What is said of them may, however, be taken as generally applicable to all lytic sera. The immune body combines with cells as is shown thus: a hæmolytic serum heated to  $55^{\circ}$  C. is mixed with its corresponding cells and the two left in contact for some time, the mixture is then centrifuged and so separated into two parts—serum and cells. If we add to the serum fraction fresh cells and complement, and incubate these at  $37^{\circ}$  C., the cells are unaffected: we conclude that immune body has been removed from the serum. If the cell fraction is added to complement, hæmolysis rapidly occurs on incubation. The cells therefore have bound the immune body and removed it from the serum. A similar experiment with cells and complement fails to show any combination between the two. The union between cells and immune body occurs at a low temperature (just over  $0^{\circ}$  C.). Once united, the combination is fairly stable, since the "sensitized" cells, even after repeated washings in saline, are hæmolysed on the addition of complement. It is, however, to some extent reversible, for if thoroughly washed sensitized cells are mixed with fresh untreated cells, all the cells present are hæmolysed by complement. Some of the immune body fixed to the first moiety of cells must have detached itself from them and become fixed to the second moiety. Complement unites with sensitized cells and produces hæmolysis—best at  $37^{\circ}$  C., and only at temperatures approaching that. It is without action on sensitized cells at  $0^{\circ}$  C. The union of complement to sensitized cells is a firm one and not at all reversible. After hæmolysis has occurred complement can no longer be found free in the mixture. This is merely a special example of the general rule that, when an antigen and antibody combine,

complement, if present, is fixed by the combination. In this case, complement plays an active part in the subsequent phenomenon—hæmolysis.

The minimum hæmolytic dose (M.H.D.) of a hæmolytic serum is the amount of serum present in the last tube showing complete hæmolysis in a series of tubes, containing constant amounts of cells and complement but decreasing amounts of hæmolytic serum, saline being added as required to make the volume in each tube the same. If the titration be repeated, using four times the former amount of complement in each tube, it is found that the M.H.D. of the hæmolytic serum is considerably less than previously. It is approximately true that the relation between the amounts of hæmolytic serum and complement necessary just to cause complete hæmolysis is reciprocal. If the amount of serum be doubled, only half the amount of complement will be needed. One difference between the mode of action of the hæmolysin and of the complement is that the absolute amount of the former is the important thing while, in the case of complement, it is the concentration and not the absolute amount which matters. A certain minimum amount of hæmolysin—that is, a definite volume of the hæmolytic serum—is necessary to sensitize a given number of red blood cells. Increasing the total volume present by adding saline to the tube has no effect on hæmolysis. If, however, the M.H.D. of complement for a tube containing a known amount of sensitized cells be found and another tube be incubated containing the same amount of complement and sensitized cells but an increased amount of saline, hæmolysis in this tube will not be complete because the concentration of complement is less.

Complement seems to act chiefly on the envelopes of the cells, injuring them so that the hæmoglobin escapes. The receptors appear to be in the envelopes since these, obtained by laking cells with water and washing in saline, are capable of binding the immune body and the two together can fix complement.

For several years a keen controversy was carried out by Ehrlich, assisted by Morgenroth, on one side and by Bordet

with his followers on the other, chiefly on the subject of the action of lytic sera. One point of dispute was on the multiplicity of complements (Ehrlich) or the unity of complement (Bordet). Each side carried out many interesting experiments to support their theories, and a number of these have practical applications of far greater importance than the theoretical points involved. None, however, had more far-reaching results than one of Bordet and Gengou, now known as the Bordet-Gengou phenomenon. *Past. pestis* was mixed with anti-plague serum and, to the mixture, complement was added. After a period of incubation, red blood cells and hæmolytic serum which had been inactivated (*i.e.* heated to 55° C. for half an hour) were added and incubation again carried out; no hæmolysis resulted. If another organism, say *Bact. typhosum*, was substituted for *Past. pestis* or another serum, say anti-cholera, was used instead of the anti-plague serum, hæmolysis occurred. This experiment strongly supported Bordet's contention that the complement involved in bactericidal action was the same as that in hæmolysis, which was the point involved. Soon, however, it was realized that the phenomenon furnished an easy, rapid and reliable method of determining whether any interaction occurred between serum and bacteria in the presence of complement. This method was much superior to that of culturing the mixture to determine whether the bacteria had been killed. The reaction is now generally referred to as the fixation of complement. It may be explained thus: [red blood cells (R.B.C.) + hæmolytic serum (H.S.)] is an incomplete hæmolytic system which acts as an indicator for the presence of complement: if complement is present, hæmolysis occurs: if not, there is no hæmolysis. In the mixture (organism + serum + complement), if the serum is specific for the organism, complement is fixed. It is therefore not free to cause hæmolysis when (R.B.C. + H.S.) is added. If, however, the serum does not correspond to the organism, complement is not fixed and, on adding the cells and hæmolytic serum, hæmolysis occurs

(Organism P + anti-P serum + complement) || + R.B.C. + H.S.  
= Complement fixed ∴ no hæmolysis.

Organism P + anti-Q serum + (complement || + R.B.C. + H.S.)  
 = Complement not fixed  $\therefore$  hæmolysis.

|| indicates an interval for incubation before (R.B.C. + H.S.) is added.

The fixation of complement reaction can be used in two ways. First, with a known antiserum the identity of an organism may be determined; second, when a known organism is used it is possible to discover whether an antibody against it is present in a serum. If a corresponding antibody is present it follows that the animal from which the serum comes has been immunized, naturally or artificially, against that organism. The reaction may be employed in the diagnosis of such a disease as typhoid fever, although in this the simpler agglutination reaction is more generally used. It has been used in other conditions, such as gonorrhœa, tuberculosis and hydatid disease.

The fixation of complement is a convenient method of indicating the combination of antigen and antibody. The reaction may be used to detect whether a foreign protein is identical with that used to produce an antiserum or not. It is much more delicate for this purpose than is the precipitin reaction and reveals the specific relation of an antibody to its antigen, even when the two undergo no reaction observable by other means.

The Wassermann Reaction is the best possible example of an excellent practical test based on false premises. Soon after the publication of the Bordet-Gengou phenomenon, Wassermann attempted to apply it to the diagnosis of syphilis. At that time the *Tr. pallidum* had not been cultured, and an emulsion of the liver of a syphilitic fœtus which was rich in spirochætes was employed as antigen. It was found that the serum of a syphilitic patient, in the presence of such an emulsion, fixed complement, while that of a healthy individual did not. Later, the antigen used was an alcoholic extract of such a liver diluted with saline, in which the various lipoidal substances present were insoluble and produced a slight turbidity. The test was found to be clinically a reliable one for syphilis and the theoretical grounds on which it was based



appeared sound. The antigen was the spirochætes contained in the liver, the antibodies were present in the patient's serum. It was, however, subsequently found that an extract of a normal liver acted equally well and that an alcoholic extract of normal heart muscle (either human or animal) was superior. Definite chemical substances (lecithin and cholesterol) could also be used as antigen, but the best results were obtained by heart extract reinforced with cholesterol. The theoretical basis of the test is untenable, but its undoubted reliability remains. Despite this, the reaction resembles any other antigen-antibody reaction, and the resemblance is increased by the finding that, when syphilitic serum is mixed with Wassermann antigen, flocculation occurs. One difficulty formerly experienced in explaining the reaction was that Wassermann antigen contained no protein and consisted of lipoids, while true antigens were all proteins. The discovery of partial antigens or haptens which, although capable of reacting with antibodies cannot cause their production when introduced into an animal's body alone, but can when introduced combined with protein, seems to offer a possible explanation of the mode of action of the Wassermann test. The theory advanced is that, in syphilis, abnormal lipoids are present in the body, derived from the breakdown of tissue; that these substances, which are haptens, in combination with some normal body protein, act as antigens; and that, in the Wassermann and flocculation tests, antibodies to them react with the lipoids to cause either complement fixation or flocculation.

The Wassermann test, whether the theory here outlined is true or not, is the most important practical application of the Bordet-Gengou phenomenon.

## CHAPTER XIX

### ANAPHYLAXIS, HYPERSENSITIVENESS

As has been seen in the preceding chapters, the tendency in immunity is usually towards the protection of the animal from injurious substances. When we inoculate an animal with a small amount of a bacterial toxin, that animal produces a substance (antitoxin), which will neutralize toxin subsequently injected, and so will protect its cells from injury. In the phenomena which we are now going to examine the element of protection seems to be absent. It is true that, as a result of the first injection of certain protein substances, some form of antibody is produced, but just as if a miscalculation had been made by nature, the effect of a second injection of the same substance is often disastrous and even fatal to the animal.

The fundamental facts of anaphylaxis are easily stated. If a guinea-pig receives an injection of a protein such as horse serum, whether intravenously, intraperitoneally, or subcutaneously and, after a period of from ten to fourteen days, another injection of the same substance, a violent reaction occurs almost immediately, frequently with fatal results. The most important point to be noticed is that the substance causing the reaction need not, in itself, be toxic. A single injection of horse serum, even in large amount, is in no way injurious to a guinea-pig, but the effects of a second injection are similar to those produced by some violently toxic substance. The animal, as a result of the first injection, after a latent period, becomes sensitive or anaphylactic towards the particular foreign protein or Anaphylactogen.

A slightly different form of anaphylaxis, known as the Arthus reaction, occurs in a rabbit submitted to repeated subcutaneous inoculations with a foreign protein, such as

horse serum. At first the injections cause little or no local reaction but, after a number have been given, reactions which are signified by the production of a local oedema occur and become more and more marked with each succeeding injection. Ultimately they lead to the formation of a firm indurated swelling in which necrosis may occur.

The anaphylactogens are all protein substances (antigens), which have not been altered by heating: horse serum coagulated by heat, for example, will not act as an anaphylactogen. The initial injection is known as the sensitizing dose; that which produces the reaction as the reacting, the anaphylactic or the shock dose.

The guinea-pig is the animal most susceptible to anaphylaxis, the rabbit and dog being much less so, while man is, luckily for ourselves, but very slightly susceptible. Much of the experimental work in connection with anaphylaxis has, therefore, been done with guinea-pigs. The dose of horse serum necessary to sensitize a guinea-pig is very small; 0.1 to 0.001 c.c. is usually sufficient, and in some instances 0.00001 c.c. was found to sensitize the animal. If another injection is made within a week the animal is unaffected. It does not become anaphylactic for at least ten days and, if the sensitizing dose was large, the necessary latent period before the anaphylactic condition is established is still longer. The reacting dose must be of considerable size—at least 100 to 1,000 times the sensitizing dose when the latter was very small. It may be administered by any route, but it is most rapid in action and a smaller amount suffices if it is given intravenously. The condition of sensitiveness, once established, persists a considerable time—in guinea-pigs for two years or more. That the reaction is due to the production of an antibody is suggested by the latent period and by the fact that the injection of a small amount of the serum of an anaphylactic or immune animal into a normal one renders the latter passively anaphylactic. Passive anaphylaxis is also found in the offspring of an anaphylactic mother.

The relation between the anaphylactogen and its antibody is strictly specific; a guinea-pig, sensitized with horse

serum, will give no reaction if human serum is subsequently injected. The exact effect of the reacting dose depends to some extent on its amount and on the route by which it is administered. An acute reaction is generally produced with rapidly fatal results when a sufficient dose is given intravenously, intracerebrally, or intraperitoneally. With the subcutaneous route, the reaction is usually less acute.

An exact description of acute anaphylaxis is difficult, since each species of animal is differently affected. Every substance causing anaphylaxis will produce exactly the same symptoms in the same species. Horse serum, egg albumin, or vegetable protein cause precisely the same phenomena in guinea-pigs, but the effects of horse serum on a guinea-pig are very different from those of the same substance on a rabbit.

A guinea-pig, almost immediately after the reacting dose, becomes restless and soon collapses with lowered temperature, urine and fæces being passed. The most marked effects are, however, on respiration, which becomes embarrassed and slowed, and finally ceases with the chest wall immobilized in the position of full inspiration. The heart may continue to beat for some time after the failure of respiration. There are spasmodic twitchings of the limbs, retraction of head and general convulsions. Death may occur within one minute of the injection, or may be delayed for ten minutes. A post-mortem examination reveals the presence of petechial hæmorrhages in the heart muscle, pleura, and intestinal walls, and explains the difficulty of respiration, since the lungs are distended and the small bronchioles are found in a condition of extreme contraction (owing to the action of the plain muscle with which they are well supplied), resulting in an almost complete occlusion of the air passages. In rabbits and dogs the outstanding feature is the great fall in blood pressure due, apparently, to abdominal vaso-dilation.

In all animals the appearances are similar to those produced by histamine and consist essentially of contraction of plain muscle and dilatation of capillaries.

De-sensitization or anti-anaphylaxis may be produced in two ways—.

1. If the animal recovers from acute anaphylaxis, it is insensitive to further injections for some time.

2. Anti-anaphylaxis may also be established by the administration of a second injection within the latent period if this injection be not given within two days of the sensitizing dose. If repeated injections are given a refractory state is developed.

The anti-anaphylactic condition is only temporary, and does not last more than three weeks in the guinea-pig. The serum of an anti-anaphylactic animal is capable, occasionally at least, of rendering a normal animal passively anaphylactic.

It is almost certain that the fundamental factor in anaphylaxis is the production of some antibody as a result of the sensitizing dose. This is not, however, the complete explanation, for although an animal may become anaphylactic passively, the state is not established immediately. When serum from the anaphylactic animal is injected, a latent interval of some hours is necessary in the case of the guinea-pig, but a shorter time suffices with rabbits and dogs. The simultaneous injection of anaphylactic serum and anaphylactogen is without effect. Further, the serum is shown not to be the only factor by an experiment in which the blood of an anaphylactic dog was removed and replaced by that of a normal animal: a typical reaction occurred on the injection of the anaphylactogen.

The theories concerning anaphylaxis are many and, for the most part, eminently unsatisfactory. Those of Friedberger, Vaughan, and Jobling and Petersen endeavour to explain anaphylaxis on the basis of the development of a toxic substance as the result of some process of digestion. They explain the similarity of anaphylactic shock produced in the same species by different proteins; but the weakness of all digestion theories is that shock occurs almost instantaneously with the injection of the anaphylactogen: there is not sufficient time for digestion to occur. The most probable theory is that of Dale, who regards anaphylaxis as a condition preceding complete immunization. If an animal

is inoculated with a foreign protein it becomes anaphylactic after a certain time. When anaphylactic, no antibodies such as precipitin can be detected in its serum. If, however, further injections are made within the latent period, immunity develops and precipitins may be detected in its serum. It is not then sensitive to further injections of the protein. The serum of an immune animal renders a normal animal sensitive after a short delay, and it is then found that precipitins are not present in the serum of the latter. Antibody freely circulating in the blood is not therefore the cause of sensitiveness, but is its antidote. Dale believes that these curious findings are to be explained as follows: As the result of the first injection of foreign protein, precipitins appear in the blood stream, but are removed from the blood subsequently and are anchored in or on the surface of tissue cells. Precipitins contained in the serum of another animal are, after the serum is injected, similarly removed from the circulation. When the reacting dose is administered a precipitate is formed in or on the cells, and it is this precipitate which causes the anaphylactic phenomenon, possibly owing to the liberation of histamine or similar substances from the cells. In de-sensitization, resulting from a dose of the protein sufficient to cause symptoms but not death, all the anchored precipitin is used up, and a further injection causes no precipitate. The thoroughly immunized animal is insensitive because free antibody is present in the blood, and this unites with the protein and prevents the latter reaching the cells. This theory seems to explain the majority, if not all, of the difficult problems of anaphylaxis, and has the further advantage of bringing it into line with the other facts of immunity.

### **Hypersensitiveness**

Hypersensitiveness is often referred to as a special form of anaphylaxis, and the two may be essentially the same; but it would appear advisable to retain the term anaphylaxis solely for the phenomena of sensitiveness induced by previous introduction of a foreign protein.

In many the injection of a foreign serum produces a

condition known as "serum sickness," in which, after a period of from several days to two or three weeks, an urticarial eruption appears with some oedema, pyrexia, albuminuria, pains in the joints, and glandular enlargement. These symptoms occur after a first injection of the serum, as also after a second. Serum sickness is a very common sequel to the administration of therapeutic serum, reactions occurring more frequently and being more marked in individuals receiving a large amount of serum intravenously than when the dose is small or when some other route is employed. The essential difference between serum sickness and anaphylaxis lies in the fact that the former may follow the first introduction of the foreign protein. It may, however, be brought into line with anaphylaxis, since it is now known that traces of foreign protein remain in the circulation for a much longer time than was formerly realized. Antibodies may have developed before the foreign protein has disappeared, and the two may interact on or in cells, so giving rise to what would be a true anaphylactic reaction. Adrenalin and ephedrine are the most useful drugs for the treatment of serum sickness.

Occasionally alarming symptoms, which may closely resemble anaphylaxis and which may have a fatal result, very quickly follow a first injection of serum. It seems impossible to explain such phenomena as has been done in the case of serum sickness, since symptoms occur almost immediately and, for the present, they must be considered as examples of idiosyncrasy.

Sensitiveness to serum (whether natural or anaphylactic) may be tested by the injection of 0.20 c.c. of the serum intradermally. In the sensitive, an urticarial patch or wheal, surrounded by an erythematous area, appears within half an hour. It is stated that desensitization can be accomplished by giving 0.025 c.c. of the serum subcutaneously and doubling the amount every half-hour, but it is rather doubtful if this procedure would succeed. If serious symptoms develop as a result of any injection of serum, adrenalin should be injected subcutaneously without delay as it usually lessens the severity of the attack.

The possibility of the occurrence of a severe immediate reaction in man following the administration of a serum should be borne in mind by the physician, but should never prevent treatment by serum in any case requiring it. The rarity of the condition is shown by the fact that, in the European War, despite the millions of doses of serum administered, only forty-nine cases of so-called anaphylaxis, with twelve deaths, were recorded among the wounded in British hospitals.

The terms *Idiosyncrasy* or *Atopy* are applied to the condition in an individual of abnormal sensitiveness to one or more of a great variety of substances which may be ingested (milk, eggs, strawberries, shellfish, quinine, and aspirin), inhaled (pollen and animal dusts), or applied to the skin (certain plants such as primula). The reactions may be associated with the respiratory tract giving rise to an asthmatic attack, the gastro-intestinal tract causing vomiting and diarrhoea, the skin producing urticaria or erythema, and the mucous membranes with excess secretion of tears and nasal mucus.

It seems advisable to group these conditions together and to separate them, provisionally at least, from anaphylaxis. Further work may, however, show the essential similarity of all these examples of hypersensitiveness as is suggested by the common occurrence of acute serum reactions and serum sickness in asthmatics who are usually hypersensitive to a number of substances, and in sufferers from hay fever (pollen-sensitive). In the majority of cases the condition of hypersensitiveness is gradually developed during life, being absent at an early age, although there is frequently an inherited tendency to hypersensitiveness. The fact that some non-protein substances against which idiosyncrasies exist, such as quinine, can act as haptens, removes one of the difficulties in considering such examples of hypersensitiveness as being special types of anaphylaxis.

The sensitivity of a person to any suspected substance can be tested either by a small intradermal injection of a solution or extract of the material or by inflicting a scratch on the skin and applying the substance. A positive result is shown



by a red flush often with a rapidly produced oedematous condition of the tissues.

In some cases desensitization may be accomplished by giving a series of subcutaneous injections of the offending substance in increasing doses.

The term Allergy which, unfortunately, is used in very different senses by different writers, is here confined to the condition of hypersensitiveness developed to some of the products of the bacteria causing certain diseases. It appears as the result of an actual infection, or more rarely follows the injection of large amounts of the bacteria themselves. Its best examples are seen in the tuberculin and other similar reactions. The mechanism of allergy appears to be quite distinct from that of the local manifestations of anaphylaxis or idiosyncrasy. In the former, the reaction is of an inflammatory nature, and in the latter vasomotor disturbances explain the resulting hyperæmia or oedema.

## CHAPTER XX

### THE CLASSIFICATION OF BACTERIA

IN Chapter I, bacteria were divided, on morphological grounds, into five classes—cocci, bacilli, vibrios, spirilla and spirochaetes. Important as morphology is, it is not the only basis of classification and, in the case of bacteria, other methods must be employed to distinguish genera and species. Unfortunately there is little general agreement as to the criteria to be adopted for this purpose. The most widely followed classification is that given in Bergey's *Manual of Determinative Bacteriology* (Fourth Edition, 1934) a brief outline of which follows.

Bacteria all belong to the Class Schizomycetes. The class is divided into Orders, the orders into Families, the families into Tribes, the tribes into Genera and the genera into Species. For the identification of a particular organism it is only necessary to give the genus and species. The genus is placed first and its name is written with a capital letter. The name of the species, which is placed second, is written with a small letter.

In the following table we give the chief orders, families, tribes and genera which contain species of medical interest. These represent only a small proportion of the whole, since medical bacteriology is only one branch of the subject. Actually almost one hundred genera are listed in the Manual.

#### CLASS Schizomycetes

ORDER	FAMILY	TRIBE	GENUS
Eubacteriales	Coccaceæ	Streptococceæ	Diplococcus
			Streptococcus
		Neisserieæ	Neisseria
		Micrococceæ	Gaffkya
	Spirillaceæ		Staphylococcus
			Vibrio
			Spirillum

CLASS Schizomycetes—*continued*.

ORDER	FAMILY	TRIBE	GENUS
	Bacteriaceæ	Chromobacteriæ	Pseudomonas
		Lactobacillæ	Lactobacillus
		Bacteriæ	Escherichia
			Aerobacter
			Proteus
			Salmonella
			Eberthella
			Shigella
			Brucella
			Alcaligenes
		Pasteurellæ	Pasteurella
		Klebsiellæ	Klebsiella
		Hæmophilæ	Hæmophilus
		Bacterioidæ	Bacteroides
	Bacillaceæ		Bacillus
			Clostridium
Actinomycetales	Actinomycetaceæ		Leptothrichia
			Actinomyces
	Mycobacteriaceæ		Mycobacterium
			Corynebacterium
			Fusiformis
			Actinobacillus
Spirochætales	Spirochætaceæ		Borrelia
			Treponema
			Leptospira

The outstanding characteristics of the various genera are given below and, as examples, the full name of one species together with the name used in this book when this differs from the one given in the *Manual*.

DIPLOCOCCUS	Gram positive cocci, usually in pairs. <i>Diplococcus pneumoniae</i> ( <i>Pneumococcus</i> ).
STREPTOCOCCUS	Gram positive cocci, in long or short chains or in pairs. Never in packets. <i>Streptococcus pyogenes</i> .
NEISSERIA	Gram negative cocci, usually in pairs. <i>Neisseria gonorrhoeæ</i> ( <i>Gonococcus</i> ).
GAFFKYA	Gram positive cocci. In animal's body usually in tetrads; in cultures in pairs or irregular masses. <i>Gaffhya tetragenæ</i> ( <i>Micrococcus tetragenus</i> ).
STAPHYLOCOCCUS	Gram positive cocci, singly, in pairs or in irregular groups. <i>Staphylococcus aureus</i> ( <i>Staphylococcus pyogenes</i> ).
VIBRIO	Gram negative, non-sporing, short, bent rods. Rigid. Single or united into spirals. Motile. <i>Vibrio comma</i> ( <i>Vibrio cholerae</i> ).
PSEUDOMONAS	Gram negative, non-sporing, motile or non-motile rods. Chiefly water and soil bacteria. Produce a green, blue or yellowish-green pigment which diffuses through the medium. <i>Pseudomonas æruginosa</i> ( <i>Pseudomonas pyocyanea</i> ).

- LACTOBACILLUS Gram positive, non-sporing, non-motile rods, often long and slender. Generally produce lactic acid from carbohydrates. Usually microaerophilic.  
*Lactobacillus acidophilus*.
- ESCHERICHIA Gram negative, non-sporing, motile or non-motile rods. Commonly occur in intestinal canal of normal animals. Attack many carbohydrates (including lactose and glucose) forming acid and frequently gas. Do not produce acetyl-methyl-carbinol.  
*Escherichia coli*. (*Bact. coli*).
- AEROBACTER Gram negative, non-sporing, motile or non-motile rods. Found in intestinal canal of normal animals. Ferment various carbohydrates (including lactose and glucose) producing acid and gas. Produce acetyl-methyl-carbinol.  
*Aerobacter aerogenes* (*Bact. aerogenes*).
- PROTEUS Gram negative, non-sporing, motile, pleomorphic rods. Produce amœboid colonies on moist media. Decompose proteins. Ferment glucose and saccharose (producing acid and gas) but not lactose. Do not produce acetyl-methyl-carbinol.  
*Proteus vulgaris*.
- SALMONELLA Gram negative, non-sporing, motile or non-motile rods. In intestinal canal of man and animals, generally producing disease. Ferment various carbohydrates, producing acid and gas. Do not ferment lactose or saccharose. Do not produce acetyl-methyl-carbinol.  
*Salmonella schottmülleri* (*Bact. paratyphosum B*).
- EBERTHELLA Gram negative, non-sporing, motile rods. In intestinal canal of man, generally producing disease. Attack various carbohydrates with production of acid but no gas. Do not form acetyl-methyl-carbinol.  
*Eberthella typhosa* (*Bact. typhosum*).
- SHIGELLA Gram negative, non-sporing, non-motile rods. In intestinal canal in man, generally producing dysentery. Attack a number of carbohydrates with production of acid but no gas. Do not form acetyl-methyl-carbinol.  
*Shigella dysenteriae* (*Bact. shigæ*).
- BRUCELLA Gram negative, short, non-sporing, non-motile rods. Do not ferment any carbohydrate. Produce disease in man and animal.  
*Brucella abortus*.
- ALCALIGENES Gram negative, non-sporing motile or non-motile rods. Do not ferment any carbohydrate. Do not form acetyl-methyl-carbinol.  
*Alcaligenes faecalis* (*Bact. alkaligenes*).

- PASTEURELLA** Gram negative, non-sporing rods. Slight powers of fermenting carbohydrates (no gas produced). Gelatin not liquefied.  
*Pasteurella pestis*.
- KLEBSIELLA** Gram negative, non-sporing, non-motile, short, plump rods. Encapsulated. Ferment various carbohydrates with acid and gas production. Aerobic.  
*Klebsiella pneumoniae* (*Bact. pneumoniae*).
- HÆMOPHILUS** Gram negative, non-sporing, non-motile, minute rods. Grow best or only in presence of hæmoglobin.  
*Hæmophilus influenzae*.
- BACTEROIDES** Gram positive or negative, non-sporing, motile or non-motile rods. Anaerobic.  
*Bacteroides bifidus* (*L. bifidus*).
- BACILLUS** Gram positive, sporing rods. Often occur in chains. Sporulation does not greatly alter shape of rods. Aerobic. Generally liquefy gelatin.  
*Bacillus anthracis*.
- CLOSTRIDIUM** Gram positive or negative, sporing rods. Rods usually enlarged at sporulation. Anaerobic or microaerophilic.  
*Clostridium welchii*.
- LEPTOTHRICHIA** Gram positive, long, thick, unbranched filaments which fragment into short rods. Anaerobic.  
*Leptothrichia buccalis* (*Leptothrix buccalis*).
- ACTINOMYCES** Branched mycelium which may break up into fragments. In lesions produce nodules in which filaments have clubbed ends. Some are anaerobic or microaerophilic.  
*Actinomyces bovis*.
- MYCOBACTERIUM** Slender, non-sporing, non-motile rods which stain with difficulty. Acid-fast. Aerobic. Slow growing.  
*Mycobacterium tuberculosis*.
- CORYNEBACTERIUM** Gram positive, non-sporing, non-motile, slender, often curved rods. Not acid-fast. Tendency to club and pointed forms. Uneven staining.  
*Corynebacterium diphtheriae*.
- FUSIFORMIS** Non-sporing, non-motile, elongated pointed rods. Stain unevenly. Anaerobic or microaerophilic.  
*Fusiformis dentium* (*F. fusiformis*).
- ACTINOBACILLUS** Gram negative, aerobic rods which may form threads and branch. Acid but no gas produced from carbohydrates.  
*Actinobacillus mallei* (*Pfeifferella mallei*).

BORRELIA	Thin, flexible spirals which are large and wavy, 3 to 5 in number. Without flagella. <i>Borrelia recurrentis.</i>
TREPONEMA	Undulating or rigid spirals. Spirals are usually close set and regular. Without flagella. <i>Treponema pallidum</i>
LEPTOSPIRA	Sharply twisted cylinders with tapering ends. One extremity curved into hook. <i>Leptospira icterohæmorrhagiæ.</i>

In this edition of the *Handbook*, we employ certain of the newer generic names given in Bergey's *Manual*, particularly those which have gained a wide acceptance. We have not followed Bergey's lead as regards certain organisms, formerly included in the old genus *Bacillus* and now, by him, distinguished by the new generic names *Escherichia*, *Aerobacter*, *Salmonella*, *Eberthella*, *Shigella*, and *Klebsiella*. We have included all these in the one genus, *Bacterium*, until such time as there is more general agreement as to how this rather unwieldy genus is to be divided.

The student should note the different meanings of *bacterium* and *bacillus* according as these words are written with initial capital or small letters. A *bacterium* is any one of the large group of vegetable micro-organisms with which this book deals, irrespective of its morphology: a *bacillus* is a rod shaped *bacterium*. When initial capital letters are used, the word *Bacterium* becomes the name of a genus consisting of Gram negative non-sporing bacilli and the word *Bacillus*, the name of a genus of aerobic sporing bacilli.

## CHAPTER XXI

### STAPHYLOCOCCI

THE name staphylococcus has been given to this group of bacteria on account of their microscopic appearance, since the cocci commonly occur in masses which have a somewhat fanciful resemblance to bunches of grapes. The various members of the group are among the commonest causes of acute inflammation with pus production in man. In general, it may be said that the lesions which they produce tend to be circumscribed rather than diffuse, but this is, of course, subject to exceptions.

#### **Staphylococcus pyogenes.**

*Staphylococcus aureus.* Rosenbach, 1884.

This organism is the most important of the group of staphylococci. The individual cocci are spherical when occurring isolated, but, when in groups, may have their adjacent sides somewhat flattened. Their average size is from 0.7 to 0.9 $\mu$ ; but exceptional forms, both larger and smaller, may be observed. Careful examination frequently reveals that the larger cocci are those which are about to divide.

In pus, the cocci occur most typically in irregular masses, which have given the name to the group; but single cocci, or those in pairs or short chains of three or four, are frequently seen. In a fresh specimen of pus from an acute lesion many of the leucocytes will be seen to have phagocytosed one or more cocci. The cocci stain well with the basic aniline dyes in aqueous solution, and retain the stain in Gram's method. *Staphylococcus pyogenes* is non-motile and does not form spores.

This coccus is easy to cultivate on any of the common laboratory media and, while it flourishes most profusely when the reaction of the food material is slightly alkaline, variations in reaction are not very important. It grows at temperatures between 12° and 43° C., the optimum being about 37° C.

On an agar plate the colony has the shape of a segment of a sphere, with smooth shining surface and even outline. Considerable variations in size may be observed, the largest having a diameter of 4 mms. There is little tendency for the colonies to coalesce, unless the plate has been very thickly spread with material, but it may be noticed that in the more crowded parts of the plate the

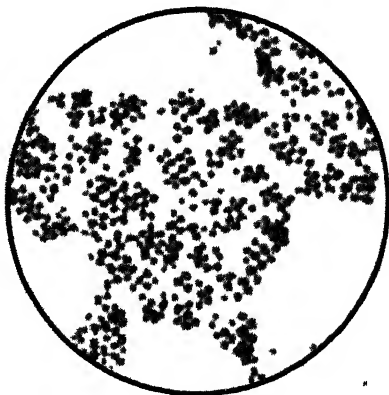


FIG. 37.—*STAPHYLOCOCCUS PYOGENES*  
FROM AGAR CULTURE ( $\times 950$ ).

colonies are very much smaller than where overcrowding does not occur. Apart from the effect of adjacent colonies, considerable variation in the size of individual colonies may be noticed, even in the same culture. The strain of the organism and the type of media in use also have a marked effect on the size of the colony. After twenty-four hours' growth the colour of the colony may be anything from white (*Staphylococcus albus*), to a deep yellow brown (*Staphylococcus aureus*). With longer incubation the colour usually becomes more intense; but this also depends on the strain examined, some strains producing pigment much more readily than others. For the development of the colour, said to be a lipochrome, oxygen is essential. Pigmentation is usually more intense in cultures grown at 25° than in those incubated at body temperature. A good pigment-producing strain, when kept in culture for some time, may gradually lose its power of producing pigment. On an agar slope, when



profusely spread, the staphylococcus gives an abundant growth with a smooth shining surface. On blood agar, the colonies resemble those on ordinary agar, except that they may be slightly larger. Surrounding each colony is a clear zone which is due to the hæmolysis and hæmodigestion of the red blood corpuscles contained in the medium. With some strains the clear zone may be very wide, with others narrow or absent. Most virulent staphylococci are hæmolytic. Cultures on solid media have a very typical odour.



FIG. 38.  
Gelatin Culture  
of *Staphylo-*  
*coccus pyo-*  
*genes* ( $\times \frac{1}{2}$ ).

*Staphylococcus pyogenes* produces an enzyme, gelatinase, which liquefies gelatin. In a stab culture, liquefaction commences around the stab and progresses until the whole of the medium becomes fluid.

In broth, the *Staphylococcus pyogenes* grows well and causes a general turbidity. If a stained preparation of a broth culture is made the cocci are found singly, in pairs, or in short chains and, but rarely, in clusters. After a few days a pellicle forms on the surface of the broth and a yellow or brown deposit of cocci collects at the bottom of the tube. Broth, free from fermentable carbohydrates, is rendered alkaline by the growth of the staphylococcus.

This staphylococcus grows well on inspissated blood serum, a proteolytic enzyme which it produces causing some digestion. It ferments a variety of carbohydrates, including lactose, glucose, maltose, saccharose, mannitol, and glycerol, without the evolution of gas. The ability to ferment mannitol is closely correlated with pathogenicity, most non-pathogenic staphylococci failing to ferment it.

This organism is slightly more resistant to adverse conditions than the majority of non-sporing bacteria. It retains its vitality for months when dried and, in this condition, may

require a temperature of 90° C. to kill it. When moist, ten minutes' exposure at 60° C. is usually fatal. Intense cold has little action, the bacteria remaining viable even after a considerable time during which they have been exposed to temperatures very much below freezing point. It is killed only after comparatively long exposures to bright sunlight. Chemical disinfectants require either longer time or stronger solutions to render it harmless than are necessary for the majority of other non-sporing bacteria.

*Staphylococcus pyogenes* produces a number of exotoxins of which the following are the most important :—

1.  $\alpha$  toxin. This toxin, when injected intravenously into rabbits, guinea-pigs or mice, kills within one hour and, when injected intradermally into rabbits or guinea-pigs, causes necrosis of the skin. The same toxin is hæmolytic, acting on rabbit and sheep cells at 37° C.

2.  $\beta$  toxin. This toxin kills rabbits, on subcutaneous or intravenous injection, after an interval of days or weeks. When injected intradermally it causes a flush without necrosis. It, too, is hæmolytic, but for the blood cells of the sheep and not of the rabbit. Hæmolysis occurs best at air temperature after previous incubation at 37° C.

3. Leucocidin, which kills human and other leucocytes.

These three toxins are best prepared by growing the staphylococci on the surface of soft agar (1 per cent.) in an atmosphere containing from 10 to 20 per cent. of CO<sub>2</sub>. The fluid obtained by mincing the agar is drained off and filtered through a Chamberland or other filter to free it from cocci.

Other soluble products of the staphylococci will be referred to later.

The virulence of different strains of the staphylococcus, as well as the resistance of its human victim, are very variable. It is the commonest cause of pustules of the skin, of boils and carbuncles, as well as of osteomyelitis. It has been found responsible for impetigo in children. It may cause abscesses of lymphatic glands, of liver, kidney and brain. It has been found as the cause of septicæmia in certain cases, and malignant endocarditis may be due to its action. It may also

cause pyæmia with multiple metastatic abscesses. It has been found alone, or associated with other organisms, in meningitis, peritonitis, empyema and cholecystitis. The tendency displayed by lesions due to staphylococci to remain localized is explained by the production, by the majority of strains, of coagulase which causes clotting of plasma. This clotting occurs in the blood and lymph vessels and also in the tissues. As a result of it, the cocci are prevented from widely invading the tissues. Later, fibrinolysin is produced which causes liquefaction of the clot. Pyæmia is due to the clotting of blood in a vessel, the invasion of the clot by staphylococci and then, as the result of partial liquefaction, the distribution by the blood stream of portions of the infected thrombus.

The action of coagulase and fibrinolysin may be demonstrated by inoculating staphylococci into citrated blood plasma and incubating at 37° C. Coagulation usually occurs within two hours and the clot is dissolved within twenty-four hours.

Many types of staphylococci produce an enterotoxin which, when swallowed, causes nausea, vomiting, diarrhœa, and collapse. This type of food poisoning usually results from the eating of some form of confectionery and particularly cakes with cream or custard filling. The toxin is produced in largest amounts in media rich in carbohydrates. It is thermostable, withstanding boiling for some time. A broth or milk culture of a food poisoning strain of staphylococcus, administered by mouth to a susceptible human volunteer or monkey, causes vomiting and diarrhœa. Intraperitoneal inoculation of kittens with similar cultures causes vomiting and diarrhœa. It is doubtful if this enterotoxin is a true toxin as antitoxin has not been prepared against it. Outbreaks of staphylococcal food poisoning are due to the growth in food of staphylococci derived either from the cow, the milk apparatus of which is infected, or from the skin of cooks.

The common laboratory animals are more resistant against this organism than is man. Guinea-pigs are very slightly susceptible, mice and rabbits more so. In the latter, a subcutaneous inoculation commonly gives rise to a local, non-fatal abscess. Moderate or large doses intraperitoneally are

usually fatal, as they also are when administered intravenously, a septicæmia or pyæmia with multiple abscesses being the immediate cause of death. In young rabbits, or in older ones which have suffered some injury to a bone, an acute osteomyelitis results from inoculation by the intravenous route. An occasional result of this method of administering the coccus is a malignant endocarditis, which usually occurs when the heart valves have been previously injured by mechanical means.

Some of the greatest successes of vaccine therapy have been in connection with the use of staphylococcal vaccines. These are particularly useful for such conditions as pustules, boils, sycosis and acne. An autogenous vaccine, administered to a patient who suffers from recurrent boils, often causes those developing to abort and may prevent the occurrence of others.

A recent advance in treatment is active immunization by injections of toxoid, prepared by treating a toxic filtrate with formalin. This leads to the production of antitoxin by the patient. This treatment is obviously only suitable for the more chronic staphylococcal lesions and, either alone or in conjunction with a vaccine, frequently gives good results. A therapeutic antitoxic serum is now available which is of definite use in the treatment of acute staphylococcal infections.

The *Staphylococcus pyogenes* (albus) is frequently described as distinct from the *Staphylococcus pyogenes* (aureus), but it is similar in almost all respects to that organism except that it produces no pigment, the cultures being pure white in colour. We are doubtful if there is any real distinction between *St. aureus* and *St. albus* since intermediates between the two form a complete series in which the colour ranges from golden brown to pure white. Further, it is possible to isolate, from a pure culture of a well-pigmented *St. aureus*, variants which would be generally classed as *St. albus*. There appears to be some connection between pathogenicity and pigment production since cultures of the more virulent strains are generally well coloured, but even more closely correlated with virulence are power of fermenting mannitol and production of hæmolysin and coagulase.

Another strain of staphylococcus (*St. epidermidis*), which is commonly found on the skin as a saprophyte, has very feeble pathogenic properties. This coccus grows more slowly than the above and produces smaller colonies. It does not ferment mannitol.

Under the name of *Staphylococcus pyogenes* (*citreus*) a staphylococcus has been described which is distinguished from *St. aureus* by the fact that its culture has a lemon-yellow colour. We are doubtful of its pathogenic powers and have never isolated it alone from a human lesion. Cocci resembling this organism are not uncommonly found as contaminants of culture media which have been exposed for some time to the air.

***Micrococcus tetragenus. Gaffkya tetragena.***

Koch and Gaffky, 1884.

*M. tetragenus* is an organism of a low degree of pathogenicity. It is found on the skin, about the mouth and nose, and in other parts of the body, apparently living as a harmless saprophyte. It is frequently found in the sputum, especially in tuberculosis of the lung.

In the body the organism is found singly, in pairs or, most typically, in groups of four (tetrads). The cocci which are about  $1\mu$  in diameter, are of round or oval shape, and are Gram positive. In body fluids a capsule is usually present. In cultures, capsules are not produced, and diplococcal forms are more common than tetrads.

The organism grows aerobically on ordinary media. On agar the colonies are grey or white, and are smaller than those of the staphylococci. Broth is clouded and a deposit may form. Gelatin is not liquefied.

In man the *M. tetragenus* has been the cause of pus production in the pleura, meninges, skin and elsewhere, and a few cases of blood infection have been recorded.

In the rabbit or guinea-pig subcutaneous inoculation may cause a local abscess. The white mouse is more susceptible, and the animal frequently dies from septicæmia as a result of subcutaneous injection of a culture.

## CHAPTER XXII

### STREPTOCOCCI

Cocci which divide in only one plane, producing chains of adherent organisms, are called streptococci. It should be realized that the term is purely a morphological one and that the species of this large genus have different cultural and biochemical characteristics and differ widely in their natural habitats and pathogenic properties.

From time to time different methods of classification have been proposed.

The earliest of these, that of v. Lingelsheim, according to the length of chain, was unsatisfactory, as this characteristic is very variable, depending, to a considerable extent, on the type of medium used.

Schottmüller used the effects which the organisms produced on blood agar as a method of classification. He distinguished three types—those producing hæmolysis, those producing a green colour and those not altering the medium. Smith and Brown modified the method slightly and distinguished  $\alpha$ ,  $\beta$ , and  $\gamma$  streptococci. The  $\alpha$  type produce, on blood agar, a greenish colour with or without a slight amount of hæmolysis; the  $\beta$  give rise to a wide zone of clear hæmolysis; and the  $\gamma$  do not alter the medium. It is now usual to distinguish only two types, hæmolytic and non-hæmolytic. The former comprise Smith and Brown's  $\beta$  type, the latter their  $\alpha$  and  $\gamma$  types, although  $\alpha$  organisms produce some hæmolysis. Non-hæmolytic streptococci are often called *Streptococcus viridans*, despite the fact that some of them do not produce a green colour on blood agar.

Many workers have attempted to classify streptococci on the basis of their fermentative capacities. On the whole these attempts have been failures, but, in a few cases, the ability or lack of ability to utilize a particular carbohydrate is a useful characteristic which is of definite value in identification.

For many years, efforts were made to classify streptococci serologically, but it is only comparatively recently that success has been attained, mainly as a result of the work of Lancefield and Griffith, who employed different methods.

Lancefield discovered that it was possible to extract from streptococci an antigenic carbohydrate which she called C substance. The C substances of different streptococci are similar in chemical composition but are antigenically different. By the immunisation of animals with streptococci obtained from different sources, she and others using her precipitation method have been able to divide streptococci (mainly, but not entirely, hæmolytic) into eleven groups which are described by letters—A, B, C, D, etc.

Griffith's method is based on agglutination of suitable cultures of streptococci by specific antisera. The antigen concerned is known as the M substance: it is a protein. Griffith worked only with hæmolytic streptococci derived from human infections.

There are, among streptococci, a much greater number of antigenically distinct M substances than C substances, and so it is now universally accepted that Lancefield's method divides streptococci into a small number of groups each of which, by Griffith's method, may be further subdivided into a large number of types.

Almost all hæmolytic streptococci causing human infections belong to Lancefield's Group A. Among these, 24 of Griffith's types may be identified. The majority of the other groups are either pathogenic only for animals or are non-pathogenic. Among them only a comparatively small number of Groups B, C, and G have been recognized as the cause of human disease. Three of Griffith's types occur in Group C and one in Group G.

### ***Streptococcus pyogenes.***

Rosenbach, 1884.

The term *Streptococcus pyogenes* is taken as equivalent to Lancefield's Group A. This organism is by far the most important of the hæmolytic streptococci found in human disease.

The individual cocci vary from 0.5 to 1.0 $\mu$  in diameter, and larger cocci, which are especially common in old cultures, are to be regarded as abnormal and involution forms. In shape they are spherical, but frequently adjacent cocci in a chain are slightly flattened. They are non-motile and do not form spores. They stain well with the ordinary aniline dyes and are Gram positive. Capsules are not usually found in films of *Str. pyogenes* prepared from cultures. By the use of very young cultures and special methods it is, however, frequently possible to demonstrate their presence, and it is probable that they are always present in virulent strains in actual infections. A variety which is spread by milk and which, in man, causes epidemic sore throat is normally well capsulated.

When a stained film of streptococcal pus is examined, the cocci are found to be present, some in pairs and some in short or long chains, each consisting of up to 20 or 30 cocci. In early lesions the diplococcal form predominates, but in pus from abscesses, long chains are common.

The *Str. pyogenes* grows on most of the ordinary laboratory media, but never so well or so luxuriantly as the staphylococci. Occasionally it is impossible to grow it in primary culture on media which have not been enriched by the addition of blood, serum or ascitic fluid. Later cultures, however, grow without these, but in any medium serum or ascitic fluid with 0.5 per cent. of glucose promotes good growth. The presence of bile in the medium inhibits the growth of the organism. The organism is aerobic and facultatively anaerobic. It grows best between 35° and 38° C., and no growth occurs below 18° C.

On agar the colonies are visible in twenty-four hours. They are circular in shape, raised in the centre, opaque and greyish in colour and, when magnified, are seen to be slightly granular. Their size rarely exceeds 1 mm. in diameter. The colonies show very little tendency to coalesce, and even when the material is very thickly spread are still discrete but very minute.

The appearance of colonies on blood-agar is very similar to that on plain agar, except that they are larger and slightly



brown in colour. Each is surrounded by an area in which the medium has become quite clear and of a pale yellow colour, owing to the lysis of the red blood corpuscles and alteration and diffusion of the hæmoglobin. The diameter of this clear area may be as much as 4 or 5 mm. Where the colonies are fairly closely associated, the adjoining zones unite and the medium in the neighbourhood is all of the clear yellow colour. (See Plate III.) Some strains of *Str. pyogenes* produce hæmolysis on blood agar only when cultures are incubated anaerobically, and with all strains, more marked hæmolysis

is shown by deep colonies than by those on the surface of the medium.



FIG. 39.—STREPTOCOCCUS PYOGENES  
FROM BROTH CULTURE ( $\times 950$ ).

In broth this streptococcus usually grows in granules, which may vary in size from those only visible with the microscope to quite large flakes. The result is that the masses of organisms fall to the bottom of the tube, leaving the upper part almost or entirely clear.

The addition of serum or ascitic fluid to the broth usually gives a much more abundant growth than would be obtained in its absence. Glucose also encourages growth, but, on account of the acid produced, growth soon ceases and the organism dies unless the amount of glucose is restricted and the medium is well buffered.

By the growth of *Str. pyogenes* in broth one or more hæmolysins are produced. Some workers distinguish two types, one of which is very easily destroyed by oxygen, while the other is oxygen stable. By the immunization of animals an antibody is produced which neutralizes the labile lysin, but not the stable one. Broth cultures also contain a leucocidin

against which an antibody has not been produced. Its relationship to the hæmolysin, if any, is uncertain. Most virulent strains produce, in broth culture, a fibrinolysin which dissolves human but not animal fibrin. This substance fails to act on the fibrin of a person recently recovered from an acute infection with the *Str. pyogenes* owing to the presence in such a person's blood of anti-fibrinolysin. Other toxins of *Str. pyogenes* will be referred to later.

Solidified serum is a very suitable medium for the growth of this coccus, good chain production generally occurring on it.

*Str. pyogenes* ferments glucose, lactose, saccharose and salicin, producing acid but no gas. It usually does not ferment inulin or mannitol. It does not hydrolyse sodium hippurate nor does it liquefy gelatin.

In all media *Str. pyogenes* tends to die readily, and an agar culture kept at 37° C. will be found to be dead in less than a fortnight. Life is prolonged by storage in an ice-chest.

The microscopical appearance of cultures depends to a very large extent on the culture medium used, the same strain of *Streptococcus pyogenes* being found in very different forms in different media. In general, in an enriched broth of correct reaction, chains of at least eight elements and frequently of twenty or thirty appear. In young cultures the chains may seem to be composed of diplococci on account of the rapid multiplication of the organisms. A broth without the addition of body fluid or one of too acid reaction may show very much shorter chains. In general, on solid media chain production is much less marked, and diplococci or chains of 4 or 6 cocci may be common. It was seen that the most characteristic microscopical preparations of *Staphylococcus pyogenes* could be obtained from solid medium: with *Streptococcus pyogenes*, broth gives the most typical picture. It is occasionally difficult to distinguish microscopically a streptococcus grown on agar from a staphylococcus grown in broth.

Man is very susceptible to invasion by the *Streptococcus pyogenes*, although individual immunity varies and strains of different virulence exist. The lesions produced are usually

less localized, tending to spread, and are more often fatal than those caused by the *Staphylococcus pyogenes*. A minute skin abrasion, infected with *Streptococcus pyogenes*, may be followed by a rapidly spreading cellulitis and lymphangitis and frequently by septicaemia. The tendency of a streptococcal infection to spread through the tissues is probably due to the production, by virulent streptococci, of an anti-fibrinogenic substance, which prevents the formation of fibrin, and a fibrinolysin which dissolves it if already formed. These substances interfere with the efforts of the body to limit the

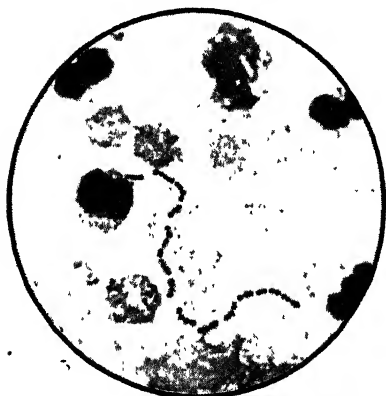


FIG. 40.—STREPTOCOCCUS PYOGENES  
IN PUS ( $\times 950$ ).

spread of infection by the clotting of the contents of blood and lymph vessels. The invasiveness of the organism is also assisted by the absence of positive chemotaxis and by the production of leucocidin which kills polymorphonuclear leucocytes. The exudate is, therefore, inclined to be serous rather than purulent, and the body may be widely

invaded before there is a satisfactory mobilization of the phagocytes. When definite pus is produced, the prognosis is better than in cases in which the exudate contains few or no cells. It is a cause of pus production in serous cavities: peritonitis, empyema and meningitis are frequently due to its action. It often produces severe pharyngitis and tonsillitis, and its presence may prove a grave complication in diphtheria. *Streptococcus pyogenes* is commonly found in acute bronchitis and broncho-pneumonia, usually associated with other bacteria but in some cases apparently pure. Osteomyelitis is less frequently due to streptococci than to staphylococci, but when the former are responsible the prognosis is very much more serious.

Erysipelas and impetigo contagiosa are among the results of infection of the skin by this organism. Puerperal fever is most to be feared when caused by the *Streptococcus pyogenes*. The most serious condition caused by it is a septicæmia, usually accompanied by acute endocarditis, which is a common termination of many streptococcal infections. The number of organisms in the blood may be as high as 5,000 per cubic centimetre, but it has been found that survival is very rare when the number exceeds 30. Blood cultures, in cases of suspected streptococcal septicæmia, should be incubated for a week before being regarded as negative as the cocci may grow very slowly.

There are, as has been explained earlier, a considerable number of different types of *Str. pyogenes*. Some of these produce large amounts of a particular toxin which is called erythrogenic or Dick toxin. This toxin acts on the skin, causing an intense erythema. When the throat of a person who is not immune to it is invaded by one of these types (particularly Griffith's types 1, 2, 3, 4, and 5), the result is an attack of scarlet fever. The same organism, in a person who is immune to this toxin, may cause a severe sore throat with complications to be mentioned later, but without the skin rash.

Scarlet fever resembles diphtheria in that, in uncomplicated cases, the organisms remain in the throat and the general effects are due to absorption of the toxin they produce. Complications may occur if the streptococci spread from the throat to other parts of the body.

The erythrogenic toxin can be prepared by growing a suitable strain of the organism in a special tryptic digest broth for 18 hours at 37° C. and filtering the culture to remove the cocci. The toxin is very thermostable, withstanding exposure to 100° C. for some time. If a subcutaneous injection of the toxin be given to a susceptible individual, he suffers from "miniature non-infective scarlet fever" with sore throat, malaise, vomiting, collapse, and a skin rash similar to that of scarlet fever. The injection of a small amount of the toxin (0.002 c.c. of filtrate diluted to 0.2 c.c.) intradermally in a susceptible person produces, in from 6 to 12 hours, a red

reaction measuring 3 cms. in diameter. This is the Dick reaction which serves to distinguish those susceptible to the erythrogenic toxin and therefore to scarlet fever from those immune to this toxin and to the disease. Immunity to scarlet fever does not necessarily imply immunity to the *Str. pyogenes* in other respects.

By the immunization of animals with erythrogenic toxin, antitoxic serum may be produced. This is useful in the treatment of uncomplicated scarlet fever and tends to reduce the subsequent incidence of complications.

Dick positive individuals may be immunized by a series of injections of the toxin. This must be used as toxoid cannot be prepared from it. Several injections, rising from 500 to 50,000 skin test doses, should be given. The skin test dose (S.T.D.) is the amount of the toxin which produces a reaction 1 cm. in diameter 24 hours after intradermal injection in the skin of a susceptible person.

It must be emphasized that there is no distinct *Str. scarlatinæ*. Any *Str. pyogenes* which produces sufficient erythrogenic toxin may cause scarlet fever in a person not immune to this toxin. In another person, it may produce erysipelas, cellulitis, sore throat, or septicæmia. The erythrogenic toxin is only one of the toxins produced by *Str. pyogenes*. It seems almost certain that some of the other toxins, not yet prepared in the laboratory, are of greater importance in infections by the organism.

The main reservoir of *Str. pyogenes* is the human throat. There it may cause tonsillitis, laryngitis, or pharyngitis, or may exist without producing symptoms. In the latter case, the individual concerned is a carrier, and it is mainly from such carriers that the organism spreads to others to cause, in them, disease of the upper part of the respiratory tract, puerperal sepsis, or any of the many other forms of infection for which it is responsible.

Many well-qualified observers believe that throat infections with *Str. pyogenes* are responsible for acute rheumatism. There is very commonly a history of a severe tonsillitis at a period varying from a few days to four weeks antecedent to

the attack of rheumatism. The most generally accepted theory is that the disease is due to an allergic condition developed against some product of the streptococci.

The larger animals are, for the most part, not very susceptible to infection by the *Streptococcus pyogenes*. Horses and sheep are very resistant, birds almost completely so. In the cow, the udder may be invaded, usually from a human source, and, although the resulting mastitis is of a very mild type, the cow may, for a long period, continue to excrete streptococci capable of causing disease in those drinking her milk. Of the common laboratory animals, guinea-pigs are least susceptible and mice most. A moderate subcutaneous injection of *Streptococcus pyogenes* in the rabbit usually produces a local abscess with a rather serous type of exudate and considerable cedema. If the dose is very large or the virulence exalted by passage, a fatal septicæmia may follow. This latter is the usual result of intravenous inoculation and the sequel of the hæmorrhagic peritonitis caused by intraperitoneal inoculation. In the mouse, by whatever route administered, a general septicæmia with death occurring within forty-eight hours commonly results. Streptococci are present in the heart's blood after death.

The virulence of any strain of *Streptococcus pyogenes* is lessened by cultivation on artificial media, but the loss is least marked if the organism has been frequently sub-cultured on medium containing blood. Virulence for any animal is enhanced by repeated passages through animals of the same species. By this procedure, however, virulence for other animals may be decreased.

Clinically, streptococci do not appear to confer any lasting immunity on those who recover from infections produced by them. That is, the same person may suffer repeatedly from streptococcal tonsillitis or erysipelas. It seems probable that this apparent lack of development of immunity may be due to the high degree of specificity of the many serological types. Immunity against one type confers little, if any, immunity against the other types.

The multiplicity of types explains the difficulty in active

immunization by vaccines and passive immunization by antisera. The toxin which has, up to the present, been most studied and against which both active and passive immunity can be produced (erythrogenic toxin) is, as compared with the other toxins, of minor importance.

The discovery of Prontosil, the first of a long series of sulphanilamides, has completely changed the prognosis, formerly so grave, in human infections by *Str. pyogenes*. These drugs have a marked effect in preventing multiplication of the streptococci in the body. For the actual elimination of the organisms, it is probable that the normal process of phagocytosis is mainly responsible.

### Other Hæmolytic Streptococci

The ten remaining groups of streptococci delimited by Lancefield's method are of much less importance in human pathology than her Group A, which is synonymous with *Str. pyogenes*.

Streptococci of Group B, constitute the species *Str. agalactiæ*. This streptococcus is the chief cause of bovine mastitis. It is not uncommonly found in the human body, in the throat and nose, vagina and fæces, but has very low pathogenic powers against man, although cases of puerperal sepsis have been ascribed to it. Its differentiation from *Str. pyogenes* is of importance, as this latter organism may be found in cow's milk and, from that source, give rise to human disease in consumers, which *Str. agalactiæ* very rarely, if ever, does. While the majority of the streptococci of this species are hæmolytic, some slightly or even non-hæmolytic strains occur. Unlike *Str. pyogenes*, it hydrolyses sodium hippurate, a useful distinguishing characteristic.

Group C appears to include a number of types of streptococci which differ in cultural and fermentative characteristics and in pathogenic powers. Among these are *Str. equi*, the cause of strangles in horses, and other animal pathogens. All are hæmolytic on blood agar. Streptococci of this group are of common occurrence in the human body, but only rarely cause disease in man.

Group D streptococci will be considered later.

Group E is represented by only a few strains isolated from cow's milk. These are not human pathogens.

Streptococci of Groups F and G on blood agar produce minute colonies and cause hæmolysis. A few human infections due to these organisms have been recorded.

No human infections have been ascribed to streptococci of Groups H, K, L or M.

### **Non-Hæmolytic Streptococci**

We propose to describe only two species of non-hæmolytic streptococci found associated with the human body. This is, almost certainly, an unwarranted simplification of the true position, but, for practical purposes, it will suffice until more general agreement has been reached as regards these organisms. The two provisional species are *Str. salivarius* and *Str. fæcalis*.

*Str. salivarius* is intimately associated with the human mouth from which it is rarely absent. It occurs in chains varying in length from six or eight cocci to hundreds. The individual cocci may be somewhat elongated and a diplococcal arrangement of the cocci in a chain is common. On blood agar, the organism grows well, producing colonies usually smaller than those of *Str. pyogenes*. In the medium, a green colour is produced together with a variable amount of lysis which is not well marked. The coccus grows well in broth producing a granular deposit. *Str. salivarius* always ferments glucose and lactose and never mannitol. Salicin may be fermented. In no case is gas produced.

*Str. salivarius* is a facultative pathogen. While in the mouth it inflicts little, if any, damage. Occasionally it invades the tissues, producing subacute or chronic disease with little pus production. Its most serious pathogenic rôle is manifested when it enters the blood-stream and, probably as a result of previous damage or congenital defect, establishes itself on a heart valve, causing subacute endocarditis.

*Str. fæcalis* (or the enterococcus) is associated with the intestinal tract and is invariably present in human fæces. It occurs either in short chains or, more commonly, in pairs.



Individual cocci are elongated, sometimes almost bacillary. On blood agar, the organism usually produces no alteration of the medium. Some strains, however, produce a green colour and others true hæmolytic. The hæmolytic *Str. fæcalis* is Lancefield's Group D streptococcus.

In broth, *Str. fæcalis* gives rise to a uniform turbidity with very little deposit. Some strains liquefy gelatin. The organism ferments glucose, lactose, and mannitol. In addition to the morphology of this organism, its most important differential characteristics are its power of growing in the presence of bile and its thermo-resistance. *Str. fæcalis* is usually capable of growth at a temperature of 50° C. and survives exposure to 60 °C. for half an hour. Like *Bact. coli*, the *Str. fæcalis* is, in the intestine, a mere saprophytic parasite but, in the tissues, it is both pathogenic and pyogenic. It is an occasional cause of puerperal sepsis. Like *Str. salivarius*, it may enter the blood-stream and cause subacute endocarditis.

While there is a widespread belief that chronic rheumatism is, in some way, connected with streptococci, and particularly non-hæmolytic streptococci, no definite proof of the connection has yet been afforded. The most plausible theory is that the tissues of a joint become sensitized to some product of the streptococci and that the disease is due to allergic reaction between the sensitized tissues and a fresh quantum of the substance.

### Other Streptococci

Many streptococci, apart from those described above, exist in a variety of habitats and may, from time to time, be isolated from the human body, particularly from the mouth, nose, throat, fæces and vagina. Among these are the lactic acid streptococci associated with the souring of milk and closely related to *Str. fæcalis*. One occasional human pathogen deserves mention. This is an anaerobic streptococcus which may cause a severe type of puerperal sepsis.

## CHAPTER XXIII

### **Pneumococcus.** *Diplococcus pneumoniae* Weichselbaum, 1886

THE pneumococcus occurs most typically as a diplococcus, each coccus being roughly oval, with one extremity pointed and the other rounded, the whole resembling a lance or the flame of a candle in shape. Usually the rounded ends of the two cocci are adjacent. Its longest diameter is about  $1\mu$ . It may also be seen singly, or in short chains of 4 to 6 cocci. Atypical forms, either spherical or almost bacillary, are sometimes to be observed. When examined direct from the body it is generally surrounded by a capsule, the total width of the coccus and capsule being about three times that of a naked coccus. The capsule rarely shows any indentation corresponding to the space between the two cocci.

The pneumococcus stains well with the usual aniline stains and is Gram positive, but dead or degenerated forms may lose the stain in Gram's method. In preparations treated with the ordinary stains the capsule does not take the colour, but can often be distinguished as an unstained halo round each pair of cocci. It may be demonstrated more satisfactorily by the methods recommended for the staining of capsules. This organism is non-motile, is without flagella and forms no spores.

The pneumococcus is aerobic and facultatively anaerobic. On artificial media its growth is never luxuriant. It requires a rich medium and may refuse to grow, especially when recently isolated, unless the medium has been enriched by the addition of blood, serum, or ascitic or hydrocele fluid. The presence of glucose in the medium also promotes growth, but the organism is soon killed by the resulting acidity, unless the medium is well buffered and the glucose restricted to 0.2 per cent. The

optimum temperature of culture is from 35° to 37° C., and no growth occurs below 22° C. On serum or ascitic agar the colonies of an encapsulated pneumococcus are somewhat larger than those of the *Streptococcus pyogenes* and are smooth. The surface is usually flat, but it may show umbilication. Adjacent colonies tend to be confluent. The colonies of old strains are small and discrete, and may be rough. On blood-agar plates the colonies cause slight hæmolysis and, in addition, produce a dark green colour. Pneumococci do not liquefy gelatin. In broth containing serum or ascitic fluid turbidity is produced, and after some days a very fine dust-like deposit of the bacteria may fall to the bottom of the tube. On milk a good growth is obtained, the milk becoming acid with or without clotting. By the growth of the pneumococcus acid is produced in media containing lactose, glucose, saccharose and, usually, inulin. During the cultivation of pneumococci, hydrogen peroxide is produced.

Microscopically, in young cultures on suitable artificial media, the typical lance-shaped diplococci are seen, together with short chains: chain formation is particularly likely to occur in broth cultures. In cultures more than one or two days old, involution forms are seen which are frequently larger than normal, atypical in shape and badly staining. Capsules, which are most noticeable in smears made from the rusty sputum of a pneumonia patient or from the blood of an infected animal, are never so well marked in culture. They are not usually produced in media devoid of body fluid. To demonstrate capsules in film preparations made from cultures, serum should be used as a diluent in place of water.

The pneumococcus has but little resistance against adverse conditions. It usually dies in less than a week on ordinary media when kept at body temperature, but may retain its vitality for considerably longer in the ice-chest particularly if light and air are excluded. It remains alive for the longest time in the blood of an infected animal which has been dried rapidly in a desiccator and stored in sealed tubes, or in the spleen of an infected animal dried and preserved in the same way. This coccus is killed in ten minutes at a

temperature of  $52^{\circ}\text{C}$ . It has little resistance against chemical disinfectants and is very rapidly killed by exposure to direct sunlight.

All pneumococci are dissolved by animal bile or by solutions of sodium desoxycholate. An easy method of testing this is to add 1 part of a 10 per cent. solution of sodium desoxycholate to 9 parts of a broth culture. For this test the acidity of the culture must not exceed a pH of 6.6. Solution may take place almost at once or after a few minutes' heating

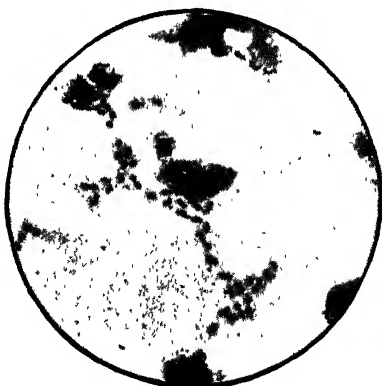


FIG. 41.—PNEUMOCOCCI IN FILM OF PUS, STAINED TO SHOW CAPSULES ( $\times 950$ ).

to  $37^{\circ}\text{C}$ . The dissolution of the pneumococci may be judged by the clearing of the broth or by the absence of cocci when the culture is examined with the microscope. This is a reliable distinction between pneumococci and streptococci, which are insoluble in bile.

The above description is true for the majority of pneumococci, except type III, sometimes called *Pn. mucosus*. This is a slightly larger coccus than the other more typical pneumococci, and is commonly oval or spherical rather than lance-shaped. It has a considerable tendency to form chains, for which reason it is sometimes classed with the streptococci, but its solubility in bile and fermentation of inulin suggest that it is a true pneumococcus. Its colonies are larger and more moist than those of other pneumococci and are sticky when touched with a platinum wire. This stickiness is explained by the organism's marked capsule development.

By the inoculation of animals with killed cultures of capsulated pneumococci, antisera against these are produced. The antigenic substance concerned is known as specific soluble substance (S.S.S.). This is, chemically, a polysaccharide and

is found in the capsules of the cocci. When a broth culture of a pneumococcus is mixed with a specific antiserum, both agglutination and precipitation occur. The former results from combination of antibody with antigen present in the intact capsules of the pneumococci, the latter from combination of antibody with the antigen which has gone into solution. There are a large number of serologically distinct types of pneumococci—32 have so far been differentiated—owing to minor chemical and, therefore, antigenic differences in the S.S.S. of these different cocci. With the exception of

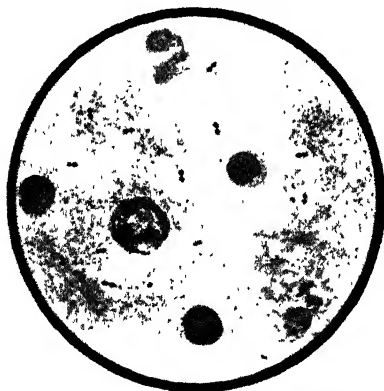


FIG. 42.—PNEUMOCOCCI IN SPUTUM  
( $\times 800$ ).

Type III, referred to above, these are morphologically and culturally indistinguishable. The capsule confers on a pneumococcus its virulence, its toxicity, and its specificity. When the capsule is lost, a pneumococcus is non-virulent and is no longer agglutinated by an antiserum prepared against it in its capsulated condition. It will stimulate the forma-

tion of antibodies, but these are group-specific and not type-specific. That is, the serum will agglutinate a non-capsulated pneumococcus, irrespective of its original type. The types are not so stable as was once believed. A pneumococcus, originally of say Type I, which has lost its capsule, may be made to adopt the capsular material of another type (*e.g.* II), and so be transformed into a Type II pneumococcus.

Almost all cases of true acute lobar pneumonia are due to the pneumococcus, sometimes associated with other organisms (*H. influenzae*, streptococci, staphylococci, etc.) or, as more commonly happens, pure. In broncho-pneumonia of children the pneumococcus is of commoner occurrence than any other single organism. The pneumococci are present in large

numbers in the "rusty" sputum typical of pneumonia. At the crisis of the disease, however, it may be difficult, or even impossible, to find them.

While pneumonia is the chief disease caused by pneumococci, it is by no means the only one. The organism is found in the fibrinous or sero-fibrinous pleurisy, so commonly associated with pneumonia, and also, sometimes accompanied by streptococci or staphylococci, in the empyema which often follows. Pneumococci are commonly present in the blood stream in severe cases of the disease. The presence of the organism in the circulation explains the fairly frequent complications and sequelæ of pneumonia—otitis media, meningitis, pericarditis, endocarditis, peritonitis and arthritis. Some of these—particularly meningitis and peritonitis of pneumococcal origin—may occur, especially in children, without an antecedent attack of pneumonia. Primary peritonitis, due to the pneumococcus, is practically confined to

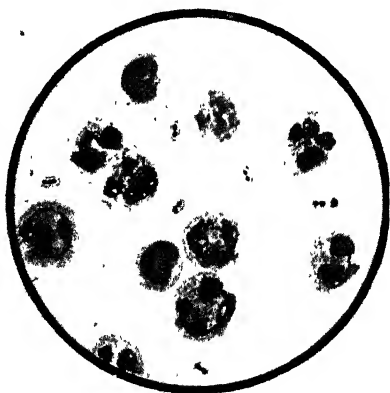


FIG 43.—PNEUMOCOCCI IN CEREbro-SPINAL FLUID FROM A CASE OF PNEUMOCOCCAL MENINGITIS ( $\times 800$ ).

female children of the poorer classes, and infection is believed to be by the vaginal route. Primary meningitis may be a very acute and rapidly fatal condition. The cerebro-spinal fluid may be turbid from the presence of enormous numbers of pneumococci, and may show practically no pus cells.

The most commonly occurring types of pneumococci in acute lobar pneumonia are Nos. I, II, III, IV, V, VII and VIII. Types I and II are responsible for more than half the cases. The disease due to Type III is more fatal than that caused by other types. Broncho-pneumonia and bronchitis may be due to one of the same types but, more commonly, are caused by other and less virulent types.

Although pneumococci are almost normal inhabitants of the mouth, throat, nose and bronchi, the majority found in health in those who have not been in contact with a case of pneumonia are either non-capsulated and non-virulent or belong to one of the less highly pathogenic types.

Pneumonia is to be regarded not as an auto-infection but as an acute infectious disease, transmitted usually from a definite case, either actually ill or convalescent, but sometimes from a healthy carrier, for persons who have been in contact with a case of pneumonia may be found to harbour fully virulent cocci of the same type as that causing infection in the patient without themselves acquiring the disease.

Man occupies towards the pneumococcus a position midway between animals which are almost or completely refractory, such as rats and cats, and those which are very susceptible as, for example, mice and rabbits. A subcutaneous inoculation of a moderate dose of a fully virulent culture into a rabbit or mouse usually produces a local oedematous condition upon which septicæmia supervenes, causing the death of the animal in from 24 to 72 hours. If a very small dose is administered, or if the virulence of the strain is lessened, only a local abscess may result. Intravenous inoculation is almost always followed by a rapidly fatal septicæmia. The same result usually succeeds the peritonitis of fibrinous or sero-fibrinous nature produced by intraperitoneal inoculation. A simple method of isolating pneumococci from sputum is by the intraperitoneal inoculation of a mouse, the organisms being recovered after death from the blood. By using a strain of correct virulence it is possible to produce lobar pneumonia in rabbits by intratracheal inoculation. If the virulence is too high septicæmia results, if too low broncho-pneumonia or only bronchitis. In monkeys, by the same technique, typical lobar pneumonia, resembling that of man, may regularly be produced.

The virulence of pneumococci is very variable: it is easily lost in artificial culture, especially in the absence of body fluids from the media. The loss of virulence proceeds parallel to the loss of capsules and corresponds with the change from

smooth to rough colony formation. It can be exalted for an animal by passage through a series of animals of that species.

Pneumonia in man is a fatal disease, not on account of the local injury to the lung, for this is not commonly the immediate cause of death, but partly by reason of the intense toxæmia which is the cause of the terminal vaso-motor collapse, and partly owing to the invasion of the blood stream. The fatality rate in pneumonia in patients with pneumococci in their blood is about 7 times that of patients with negative blood culture. The pneumococcus does not produce exotoxin in artificial cultures and the chief toxic product is probably the capsular material.

Antisera are now available against the more commonly occurring types of pneumococci. When potent antisera are administered in adequate doses the severity, the duration, and the fatality of both lobar and broncho-pneumonia are considerably reduced. Certain of the sulphanilamides have also a very definite therapeutic action. The most widely accepted opinion at present is that, for the maximum benefit to the patient, both specific antiserum and sulphanilamide should be administered. The same combined treatments should be used in other pneumococcal infections and particularly in pneumococcal meningitis. The antisera are both antitoxic and opsonic and, in this connection, it should be remembered that the leucocytes are man's chief defence against pneumococci. A marked leucocytosis (30,000 or more per c. mm.) is to be regarded as a favourable sign in pneumonia, while a leucopenia warns us that the prognosis is grave.

An adequate dose of therapeutic antiserum is 10,000 to 20,000 units. This should be repeated every 8 to 12 hours until the temperature falls below 102° F. The Felton unit used for anti-pneumococcus serum was originally fixed as the amount which prevented the death of a mouse when administered subcutaneously with one million lethal doses of pneumococci.

For the determination of the type of pneumococcus present in pneumonia the ordinary methods of plating and sub-culturing are too slow to be very useful. One method is



to inject a mouse intraperitoneally with a small amount of sputum which has been well washed and then emulsified in sterile saline. In 5 to 8 hours the peritoneum contains a strong and fairly pure growth of pneumococci. It is washed out with saline, the washings being centrifuged slowly to deposit the cells and masses of fibrin, and then rapidly to deposit the bacteria, which are re-suspended in saline and tested against the type agglutinating sera, a film being made to test the purity of the culture. A much more rapid method is that of Neufeld. A loopful of sputum is mixed, on a cover glass, with two or three loopfuls of type serum and one loopful of methylene blue stain. From this a hanging drop preparation is made. If the pneumococci are of the same type as the antiserum, the capsules of the pneumococci are seen to be swollen and much more sharply outlined than in a preparation containing a different type antiserum.

## CHAPTER XXIV

**Meningococcus.** (*Micrococcus intracellularis meningitidis*.)  
*Neisseria intracellularis.* Weichselbaum, 1887

THE meningococcus is the causal organism of cerebro-spinal fever of both epidemic and sporadic types. In the cerebro-spinal fluid from a case of meningitis, due to this organism, many of the cocci may usually be seen. They lie chiefly within the polymorphonuclear leucocytes, but, especially in the early stages of very acute cases, many may be free from cells, which are often few in number. Most typically they are found as diplococci, the cocci being somewhat flattened on their adjacent sides, which gives to the individual somewhat the shape of a coffee-bean. Although diplococci are the commonest form, single cocci or small groups, particularly groups of four, are also seen. There is a very great variation in the size of meningococci, even in the same preparation, some being very minute and others relatively large. The largest may have a diameter equal to five or more times that of the smallest, the average size being about  $0.8\mu$  in diameter. They stain easily with the ordinary aniline dyes; but it is frequently observed that some individuals take the stain much more intensely than others. They are Gram negative, non-motile and do not form spores.

The meningococcus, while not very difficult to cultivate, demands a rather rich medium. When taken fresh from the body, no growth may be obtained on plain agar, but on agar containing blood, serum, or ascitic fluid, good growth occurs. The presence of 0.5 per cent. of glucose also improves the growth. Freshly isolated strains will not grow below  $23^{\circ}\text{C}$ . and only feebly at that temperature. Optimum growth is obtained at  $37^{\circ}\text{C}$ . It is a strict aerobe.

On serum or ascitic agar the colonies are flattened, with a smooth shining surface, of a white colour with a slightly grey or blue tinge and about 2 mms. in diameter. Their centres are very finely granular and resemble ground glass, but towards their edges, which are smooth, they are almost transparent. When touched with a platinum wire they are found to be rather viscid, but emulsify easily in saline. The meningococcus also grows well on Löffler's serum. In serum-broth it produces a slight turbidity and occasionally a pellicle forms: after a few days a deposit falls to the bottom of the tube. The meningococcus ferments glucose and maltose but is without action on saccharose.

Films made from cultures resemble fairly closely those prepared from cerebro-spinal fluid, but the occurrence of the diplococcal form is not quite so regular. The irregularity in

size and in staining is even more marked, and in preparations made from cultures 48 hours old many large, badly staining involution forms are seen.



FIG. 44.—MENINGOCOCCI IN PURULENT CEREBRO-SPINAL FLUID.

This organism has very little resistance against adverse conditions, and cultures kept at 37° C. may be found to be dead in 3 or 4 days. It is rapidly killed by drying, by exposure to heat or cold or to sunlight.

By the inoculation of animals it is possible to prepare agglutinating sera against meningococci. Clear cut results are rather difficult to obtain, but it appears that the majority of meningococci may be assigned to one of two groups, I and II. Group I includes the earlier described Types I and III and Group II, Types II and IV. Agglutination is best carried out by using fairly concentrated sera at a temperature of 50° C.

for 24 hours. Any Gram negative diplococcus (apart from the gonococcus and *N. catarrhalis* which have been described as occurring in this situation) which is found in the cerebro-spinal fluid may rightly be called a meningococcus, even if it fails to agglutinate with either of the group sera.

The chief disease produced in man is meningitis, involving either the meninges of the brain or of the cord, but more usually of both. This condition may be very acute, with death occurring within 24 hours, or may be subacute or almost chronic. The cerebro-spinal fluid is under considerable pressure, and may be distinctly turbid from the large number of cells (almost entirely polymorphonuclear leucocytes) contained in it. The diagnosis is usually easy, either microscopically or by culture. In making cultures from the fluid, a considerable amount should be used, or it should be centrifuged and the deposit employed, as many of the cocci present are dead and so do not produce colonies.

Blood cultures have revealed meningococci in the blood stream in a considerable proportion of cases of meningococcal meningitis, and, in some cases, before symptoms of meningitis were detectable. The organism has also been found in arthritis, in pericarditis, in the purpuric patches of the skin, and in the urine in association with meningitis, but these are of rare occurrence. Apart from the meninges, its most important site is in the naso-pharynx, where it produces a rhino-pharyngitis. It is there found in many persons who have not had and who do not develop meningitis. On account of the very slight powers of resistance of this organism, one is forced to believe that the spread of the disease is direct from man to man, the organism passing from the naso-pharynx of one to the naso-pharynx of the other. The method by which the organisms pass from this situation to the meninges cannot yet be regarded as certain—it may be by the blood stream, from which the organisms pass to the ventricles by the choroid plexus, by the lymphatics or by the olfactory nerves. Nor do we know why one man with meningococci in his nasopharynx acquires meningitis while another does not. We can only say that it is a question of individual

susceptibility or of individual resistance. We know that the proportion of meningococcal carriers is higher among contacts than among non-contacts, and that it rises in the winter and spring months (at which times epidemics are commonest), particularly among people living under conditions of overcrowding. The duration of the carrier state is not usually long—a few weeks—but it may persist obstinately for months. It is not yet agreed whether it is worth while isolating meningococcal carriers or not. It would seem better to take steps to prevent overcrowding, particularly in dormitories and barracks.

The meningococcus possesses but a low grade of virulence for animals. By intracerebral or intrathecal injection of young cultures in certain types of monkeys an acute meningitis has been produced. Intravenous or intraperitoneal inoculation of mice or guinea-pigs may give rise to a fatal septicæmia or to death without septicæmia. The virulence of the organism for animals is greatly increased by the addition of mucin to the inoculum. It is noteworthy that some strains of meningococci may cause death in these animals, even when dead cultures have been administered, and that the fatal dose of the dead and living cocci may be very little different. This points to the existence of a powerful endotoxin in the meningococci. No exotoxin has been demonstrated in cultures.

By the progressive inoculation of cultures into horses, therapeutic sera of great value have been made. These should be administered at the earliest possible moment in the disease. The intrathecal route is usually employed, but it is doubtful if this has sufficient advantage over the intravenous and intramuscular routes to balance its disadvantages. At first polyvalent serum (prepared against both groups) is used. As soon as the responsible group of coccus is ascertained, serum of this group should be employed. Serum should be administered daily until convalescence has been established.

The antibodies in the serum probably act chiefly as opsonins, for it is mainly by phagocytosis that meningococci are destroyed. As in the case of pneumococcal infections, the combination of antiserum and one of the sulphanilamides is

more effective than either alone, and the introduction of these drugs has greatly improved the prognosis in cerebro-spinal fever.

In a considerable proportion of cases a diagnosis of meningococcal meningitis may be made by a precipitin reaction performed by layering the centrifuged cerebro-spinal fluid on top of anti-meningococcal serum in a tube which is kept for one hour at 37° C. A positive reaction (appearance of ring of precipitate) indicates the presence in the fluid of products of the meningococcus. A similar method, using group serum, may indicate the group of the infecting organism.

The identification of the meningococcus in the cerebro-spinal fluid presents no difficulty. In cultures taken from the nasopharynx, considerable trouble is experienced, for a number of other Gram-negative diplococci are frequently found in that situation. The chief of these are *N. pharyngis* and *N. catarrhalis*. The colonies of *N. pharyngis* are white or yellow, very cohesive and may be picked up whole when touched with the needle. This organism grows well on plain agar at 23° C. and ferments glucose and maltose and frequently saccharose. The colonies of *N. catarrhalis* have a much closer resemblance to those of meningococcus. They are, however, more opaque with slightly irregular edges and are definitely white. The organism may grow at 23° C., but often only feebly. It has no fermentative action on glucose, maltose, or saccharose. *N. catarrhalis* is frequently associated with acute and chronic inflammations of the respiratory tract. It is probably not the primary infecting agent in these conditions, but an important secondary invader. It is almost devoid of virulence for animals.

## CHAPTER XXV

### Gonococcus. *Neisseria gonorrhœæ* Neisser, 1879

THE gonococcus is an organism which resembles the meningococcus very closely, in microscopic appearance and in cultural characteristics. It is most commonly found in the body as a diplococcus, the size of a pair of cocci together being about  $1.5\mu$  by  $0.8\mu$ . The cocci are either flat or, more usually, somewhat concave on their adjacent sides: in the latter case

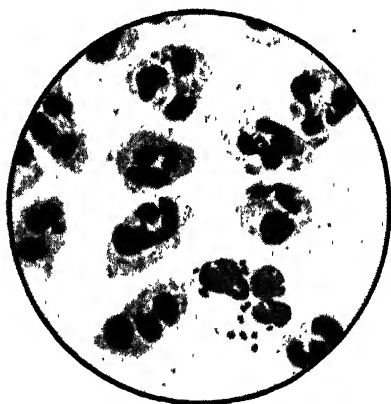


FIG. 45.—GONOCOCCI IN PUS ( $\times 950$ ).

a small oval unstained area is visible between them. They have been described as resembling a pair of kidneys or beans placed together with their concave borders in apposition. In stained films of the pus obtained from the urethra in acute gonorrhœa the great majority of the cocci are seen to lie within leucocytes. (See Plate I.) As many

as one hundred cocci may occasionally be seen within a single pus cell. It is remarkable that often, so far as can be determined by microscopical examination, neither the cocci nor the pus cells have been injured by this invasion, both taking the stain normally. The nucleus is never invaded by the cocci. In the very early stages of gonorrhœa, when the discharge is mucoid rather than purulent, very many gonococci

may be seen outside pus cells, and a similar picture may be obtained in an old standing case of gleet, except that in this condition the cocci may be very scanty. The gonococcus takes the common aniline stains well, but is Gram negative. For diagnostic purposes, Gram's stain should always be used but, if the number of gonococci be few, they may easily be overlooked. By using Pappenheim's stain as the counter-stain in Gram's method, the advantage of a contrast in colour between the cocci (red) and the cells (blue) is secured without sacrificing the diagnostic value of Gram's method.

The gonococcus is a very delicate organism and difficult to grow: it is aerobic. The most satisfactory medium for the growth of the gonococcus is agar containing unheated blood, serum, or ascitic fluid. The material should be transferred immediately from the patient to the medium, which should be incubated without delay; a considerable amount of pus should be used. The agar should be one which is not too stiff. The surface should be moist and water of condensation should be present. Cultures usually succeed best in an atmosphere containing 10 per cent. of CO<sub>2</sub>. Drying of the medium must be prevented. The temperature should not exceed 37° C.; probably 36° is better.

Colonies usually appear within 24 hours, but may be delayed for 48 hours or even longer. The colonies vary in size, but are rarely more than 2 mms. in diameter and are usually smaller. They are semi-transparent and of a greyish-white colour. In shape the colonies are round and their margins are often scalloped or undulating. Later the centre of the colony becomes rather more opaque and granular. The colonies are usually discrete and have a somewhat viscid consistency.

In serum-broth only a slight growth is obtained, the medium becoming faintly turbid; after a few days the medium becomes clear, partly owing to the cocci falling to the bottom of the tube and partly owing to autolysis. Occasionally a pellicle forms on the surface. The gonococcus ferments glucose but not maltose or saccharose. The microscopic appearance of gonococci in culture resembles that seen in direct films, but there is much greater irregularity in size, swollen and badly



staining degenerated cocci being present, and the diplococcal form is accompanied by single cocci or tetrads.

The gonococcus has very slight powers of resistance. It dies in culture in a few days at 37°C., and even more rapidly at air temperature or in the ice-chest. It is killed by exposure to a temperature of 42° C. for some hours. Desiccation, exposure to sunlight or to weak disinfectants kill it rapidly. It has, however, been found to survive as long as several weeks when dried in a thick layer of pus.

By the inoculation of animals agglutinating sera may be produced. There are a considerable number of distinct serological strains of gonococci. This fact has to be allowed for in making use of complement fixation tests for diagnosis and in using vaccines for treatment.

Man is the only animal naturally infected by gonococci. Infection is, on account of the delicacy of the organism, almost always direct from individual to individual. The gonococcus attacks chiefly the urethra, both in the male and female, producing an acute catarrh. The cocci quickly penetrate the surface, passing between the epithelial cells which are loosened and desquamated, and invade the tissues as far as the superficial layers of the submucous connective tissue. There is an energetic emigration of polymorphonuclear leucocytes, and the discharge, at first mucoid, soon becomes purulent. The disease spreads and, in the male, if untreated, the prostate may be involved and occasionally the bladder. Orchitis and inflammation of the cord and epididymis are also of fairly frequent occurrence. In the female the urethra is the most common starting-point, and later the cervix is involved. In adult women the vagina usually escapes, but a severe vulvo-vaginitis occurs in young girls. In this case the disease is not generally venereal, but is due to the use in common of utensils or clothes, especially in children's hospitals and schools in which the condition may become endemic. The disease in the woman often involves the glands of Bartholin and, more rarely, the uterus, producing an endometritis. The gonococcus is a common cause of pyosalpinx with subsequent sterility and of localized peritonitis. Occasionally, in either sex, the

mucous membrane of the rectum and anus may be involved. Gonococcal conjunctivitis may occur at any age, but particularly in the new-born, from infection during parturition. The resulting ophthalmia neonatorum, if neglected, is a frequent cause of blindness. Gonococcal septicæmia, with or without endocarditis, occurs as a complication of gonorrhœa, as also does arthritis.

The gonococcus is almost without pathogenicity for the lower animals. In mice or guinea-pigs intraperitoneal injection of gonococci may produce a mild peritonitis. This is probably due chiefly to the endotoxins of the organism, as suspensions of killed gonococci are almost as effective. Exotoxins have never been satisfactorily demonstrated; but it should be noted that the gonococcus undergoes autolysis rapidly, so a broth culture even a few days old will, after filtration through a porcelain filter, contain the endotoxins of many cocci. Inoculation of the human urethra with endotoxin causes an acute catarrh.

As a result of an attack of gonorrhœa very little, if any, immunity is established. The disease, however, may exist in a quiescent form in which the discharge is very scanty or indeed absent. A person in this condition may infect another in coitus, or the disease may suddenly become active in himself, often in response to some stimulus such as an alcoholic debauch.

A point of practical importance, both in prophylaxis and treatment, is the susceptibility of the gonococcus to silver salts such as argyrol and protargol in concentrations which do not harm the mucous membrane.

The bacteriological diagnosis of acute gonorrhœa in the male is not difficult, as the microscopic picture in films of pus from the urethra is so characteristic. Gram's method of staining, it should be emphasized, is the only reliable one for diagnostic purposes. A diagnosis can never be given in any case unless typical grouping of Gram negative diplococci is seen within a cell. In the female, diagnosis is less easy. Pus from the urethra, cervix, gland of Bartholin or clitoris should be obtained: vaginal swabs are practically useless. Since other Gram negative cocci are frequently

found in the vagina, the criteria mentioned above must be satisfied. When pus is scanty its amount may be increased, and also the number of gonococci, in various ways. The chief of these are excess of alcohol, injection of silver nitrate solution into the urethra and the use of a vaccine in sufficient dose to obtain a marked local reaction. In other parts of the body the diagnosis is even more difficult, on account of the possible presence of meningococci and other very similar cocci. If possible, cultures should be obtained and the gonococcus identified by its various cultural characteristics and by its fermentation of glucose only. Apart from microscopic and cultural methods, a diagnosis of gonorrhœa may sometimes be made by the complement fixation test. This is uncertain in acute cases, but in the late complications of the disease, such as arthritis, it is of considerable value. On account of the many types of gonococci it is essential that the antigen should be fully polyvalent.

Anti-sera have been tried in the treatment of the disease, but have not met with success. Vaccines are useful, not so much in acute gonorrhœa as in its complications. In using vaccines, the first dose should be small as severe reactions may follow large doses.

Some of the sulphanilamides have conclusively proved their value in the treatment of gonorrhœa. It is not yet quite clear whether they should be administered early in the disease or withheld until antibodies to the cocci have developed.

## CHAPTER XXVI

### ***Bacillus anthracis.***

Koch, 1876.

THE *B. anthracis* shows considerable variations in size, being usually  $5-10\mu$  by  $1-2\mu$ . It is found, in the lesions which it produces in animals and man, singly, in pairs, or in short chains, which rarely consist of more than five individual bacilli. Under natural conditions it is surrounded by a transparent capsule, which may be observed as a rather badly defined, unstained area around the bacilli. This may, however, be seen better when special capsule stains are used. The adjacent ends of bacilli in chains appear either to be sharply cut across or slightly dimpled. The result of the latter form is that an oval unstained area separates the bacilli. The capsule is continuous over the whole chain. The organism is non-motile, and spores are never seen in preparations made from material immediately it is removed from the body. The bacillus stains easily and is Gram positive. A certain amount of care is, however, necessary in decolorizing in Gram's method lest some of the bacilli lose the stain.

The anthrax bacillus is aerobic and facultatively anaerobic. The organism grows well on all ordinary media at temperatures between  $19^{\circ}$  and  $40^{\circ}$  C. Growth ceases below  $14^{\circ}$  and above  $44^{\circ}$  C.

The colonies on agar are of a greyish white or cream colour. They have a rather solid opaque centre with an irregular, wavy, less opaque margin. When more closely examined with a lens the colony is found to be made up of tangled threads, somewhat resembling matted locks of hair or a tuft of cotton wool. The colony is so characteristic that, once seen, it can readily be recognized again. With the low power of the micro-

scope it is found that the strands or threads which make up the colony are each composed of a number of parallel chains of bacilli.

On a gelatin plate the colonies resemble those on agar, but after a few days' growth liquefaction commences. In gelatin stab-culture a characteristic appearance is sometimes, but not always, seen. A white line of growth shows the track of the needle, and from this radiate, in all directions at right angles to

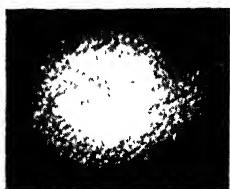


FIG. 46.—COLONY OF  
B. ANTHRACIS ON  
AGAR ( $\times 10$ ).



FIG. 47.—EDGE OF COLONY OF B.  
ANTHRACIS ON AGAR ( $\times 75$ ).



FIG. 48.—B. AN-  
THRACIS IN  
GELATIN ( $\times \frac{1}{2}$ ).

the central stem, fine branches which are longer in the upper part of the medium than below, the whole somewhat resembling an inverted fir-tree. Liquefaction commences at the top and gradually spreads downwards.

In broth, a pellicle sometimes forms on the surface, but the bacilli more usually grow in flocculent masses, which sink to the bottom of the tube, leaving the upper part of the broth

almost clear. The bacillus ferments glucose, maltose, and saccharose without gas production.

The microscopic appearance of anthrax bacilli in culture is different in many respects from that of bacilli removed from an animal's body. In artificial media very long chains of bacilli occur. Capsules are not formed unless the medium is fluid serum and, even in this, their presence is not constant.

After a few days' growth, or even in 24 hours in some cases, a small refractile spot is to be seen in the centre of many of the bacilli when they are examined unstained. In stained preparations this spot may appear to be at first a less intensely stained part of the bacillus.

After a short time its size increases, until it is seen to be a definitely outlined, unstained body situated at about the centre of the bacillus. Gradually the bacillus disintegrates and this body, now the fully formed, oval spore, is liberated. The spore is uncoloured by ordinary stains, but may be demonstrated by one of the special spore stains. The object of the

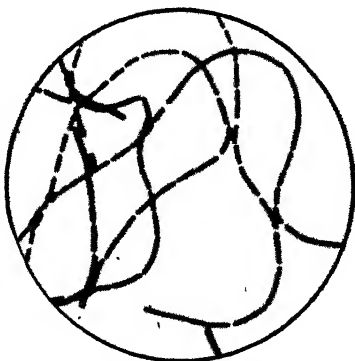


FIG. 49.—*B. ANTHRACIS* FROM AGAR CULTURE ( $\times 750$ ).

formation of spores by anthrax bacilli is to preserve the race in adverse circumstances. As has already been mentioned, spores are never found in the body of an animal during life. Further, free oxygen is necessary for spore formation in artificial culture. Growth of bacilli may occur at temperatures as low as  $14^{\circ}$  and as high as  $44^{\circ}$  C., but spore formation ceases below  $16^{\circ}$  and above  $42^{\circ}$  C. In fact, by prolonged cultivation of the bacilli at  $42^{\circ}$  to  $43^{\circ}$  C., or by growing on a medium containing phenol, a strain is produced which may never regain the power to produce spores. The explanation of these isolated facts concerning the spore

production of anthrax bacilli is as yet unknown, but the facts themselves are of considerable practical importance in dealing with the disease.

The resistance of the vegetative form of anthrax bacilli is less than that of many non-sporing bacteria. Ten minutes' exposure in the wet state to a temperature of 55° C. is sufficient to kill the bacilli, and their resistance to disinfectants is not marked. The spores, particularly when dried, are very refractory to adverse circumstances and may remain viable for many years. Three hours' exposure to a temperature of 140° C. may be necessary to kill them, but when wet ten minutes at boiling temperature is sufficient. The power of resistance of different strains against disinfectants is very variable. Growth has occurred from spores which had been in contact with a 5 per cent. solution of phenol for 14 days. Formalin is one of the most potent disinfectants against anthrax spores, and is largely employed in the treatment of infected animal products such as wool and hides.

The natural disease affects herbivora—chiefly sheep and cattle—in which the mortality may be as high as 80 per cent. Horses, goats, and pigs are less commonly attacked. Elephants are susceptible. In acute cases, the animal may appear in customary health until a short time before death, when convulsions, rigors and elevation of temperature occur. The most marked post-mortem finding is the great enlargement of the spleen, from which fact the name Splenic Fever has been given to the disease. The spleen, which is of a dark red colour, is very soft and friable, and is found to contain enormous numbers of bacilli. The liver and kidneys may also be enlarged and congested. In these organs bacilli are present, chiefly in the capillaries. The lungs are congested and may show catarrh. The lymphatic glands, especially in the mediastinal and mesenteric groups, are enlarged and surrounded by an oedematous zone. The intestines, which are usually very markedly congested, show a loosening of the epithelium and contain a bloody fluid. The blood, in which bacilli are present in large numbers, is dark in colour and does not readily clot. There can be little doubt that infection in

animals is chiefly by the ingestion of spores (the bacilli being destroyed by the gastric juice), and yet feeding laboratory animals with spores rarely causes infection. It has been suggested that the consumption of food which inflicts damage to the mucous membrane is essential. If, for example, thorns were ingested with anthrax spores, the damage inflicted by the thorns might be sufficient to allow the spores to pass into the wall of the gut, where they might develop into the vegetative form and be carried by the lymph and blood to other parts of the body. Although bacilli are carried by the blood, they do not multiply in it until shortly before death. The blood appears to possess considerably higher powers of resistance than the tissues of the body, and it is only when the animal's resistance is completely broken down that a true septicæmia occurs. As has been said above, spores are not found in the body, but before death there are commonly bloody discharges from the mouth, nostrils or anus and these contain anthrax bacilli. On the ground the bacilli may assume spore form, and so the pasture is infected, and may remain in this condition for many years. If the animal's body is deeply buried unopened, the bacilli in it, being without oxygen, cannot spore and, in consequence, rapidly die. Only where a post-mortem examination has been performed is there any great danger of spreading the disease from a dead body. Animals may also be affected with the cutaneous form of anthrax similar to that of man, but this variety is much less common than the preceding.

Man occupies a position as regards resistance higher than such animals as sheep and cattle, but lower than dogs, cats and birds, which are relatively insusceptible. He may be infected by the cutaneous, respiratory or intestinal routes. Skin infection commonly occurs in farmers, veterinary surgeons, butchers, or those whose work is in connection with hides, hair or wool; but infection has also resulted from the use of shaving brushes, the bristles of which had come from infected animals. The spores or bacilli gain entrance through cuts or abrasions or possibly by way of the hair follicles. The face and neck, hands, arms or back are the usual sites for the characteristic lesion, which is known as the "Malignant Pustule." From



one to three days after infection a small papule, usually painful, occurs. This soon becomes vesicular, the contained fluid being clear or blood-stained. The centre becomes necrotic and black in colour and around it a ring of vesicles forms: the whole area is congested and oedematous. The lesion may then undergo healing; but often, if untreated, the area of oedema increases, the lymphatic vessels and neighbouring glands become swollen and the blood stream is invaded. The fatality rate in man is from 5 to 15 per cent. Once a definite septicæmia has been established death is the rule.

The respiratory form of anthrax—Woolsorter's Disease—is due to the inhalation of dust from infected wool, hair or hides. The parts primarily affected are the air passages, especially the large bronchi. In the mucous membrane swollen patches are seen, and these soon become necrotic, leading to the production of small ulcers. The surrounding tissues are congested and oedematous and the bronchial and mediastinal lymphatic glands are enormously enlarged and engorged. The lung tissue itself shows similar congestion, and much oedema is also frequently present. Pleural and pericardial effusions are formed and a state of general septicæmia precedes death which occurs in practically all cases.

The intestinal form, which is rare in man, may be due to the eating of imperfectly cooked infected meat or the swallowing of spore-containing dust. It is characterized at first by hæmorrhagic lesions in the intestinal mucous membrane and the appearances, in the later stages, resemble fairly closely those found in the disease in sheep.

The smaller laboratory animals—mice, guinea-pigs and rabbits—are susceptible, in the order named, to anthrax infection as a result of subcutaneous inoculation. This fact allows us to judge the virulence of a given strain of anthrax bacillus with considerable accuracy. If fully virulent, it will kill all three animals; if of moderate virulence, mice and guinea-pigs but not rabbits will succumb; while if the virulence is low only the mouse will die. A strain almost or completely devoid of virulence fails to cause the death of any of the three animals. As a result of subcutaneous inoculation of fully

virulent bacilli the animal dies within 72 hours. There is an oedematous and hæmorrhagic infiltration at the point of inoculation and the spleen is enlarged and congested. The kidneys, as also the other internal organs, are congested and hæmorrhagic spots are frequently present in the serous membranes. Bacilli are few in the blood until just before death, but after death may be found in large numbers in the heart's blood. Very many are present in the spleen: in the liver and kidney fewer are found and these chiefly in the capillaries. The tissue of the body most susceptible to infection with *B. anthracis* is, apparently, the skin.

The precise cause of death in anthrax is as yet uncertain. There may be a mechanical factor from capillary blockage, but this cannot be the chief one. The disease has the appearance of an intense toxæmia, but toxins, either endotoxins



FIG. 50.—*B. ANTHRACIS* IN SECTION OF KIDNEY OF INFECTED GUINEA-PIG ( $\times 400$ ).

or exotoxins, have never been satisfactorily demonstrated. The virulence of different strains of anthrax bacilli is very dissimilar. As with the majority of bacteria, artificial culture lowers virulence, but much more slowly than with most other organisms. A strain which has lost its power of producing capsules has usually also lost its virulence. Virulence can be lowered fairly rapidly in a number of ways. Pasteur, by culturing the bacilli at a temperature of  $42^{\circ}$  to  $43^{\circ}$  C., produced a strain of non-virulent bacilli. Loss of virulence is also attained by the addition of small amounts of phenol or other antiseptic to the culture medium. Virulence can be exalted by passage through susceptible animals.

Prophylaxis, so far as man is concerned, is a matter chiefly of industrial hygiene. In the case of the herbivorous animals, ordinary hygiene is impotent, since the sterilization of

infected pastures is impossible. It was found that vaccines of dead bacilli were useless in conferring immunity on animals. Pasteur, therefore, sought to immunize sheep and cattle with living cultures of lowered virulence. After 24 days' growth at 42° C. an originally virulent culture was not fatal to the guinea-pig or rabbit but killed a mouse. A 48-hour broth culture of this attenuated bacillus grown at 37° C. constituted his "Première vaccine." After only 12 days at the high temperature the strain was virulent for the guinea-pig as well as the mouse. A broth culture of this was the "Deuxième vaccine." The vaccines were injected subcutaneously at an interval of 12 days. In about 1 per cent. of the inoculated animals death from anthrax occurred; but this was preferable to the very high mortality from the natural disease, at that time widespread throughout France. The immunity thus produced lasts about one year. Besredka claims that these vaccines are more effective if introduced intradermally instead of subcutaneously. Other methods, particularly by a combination of vaccine and antiserum, have also been successful.

By the repeated inoculation of large animals first with attenuated and later with fully virulent cultures of anthrax bacilli a potent antiserum is produced. The most successful of the antisera is that of Sclavo, who injected asses, first with antiserum and slightly attenuated cultures, later repeatedly with cultures which were fully virulent. This serum, when administered to the extent of 30 to 40 c.cs. intravenously or intramuscularly or around the lesion in cases of malignant pustule, has had great success, whether the pustule has been excised or not. There can be no question of its utility, but its method of action is uncertain. It may contain antitoxins, but no toxins have yet been demonstrated in cultures of *B. anthracis*. It does not appear to be either bactericidal or bacteriolytic. Most probably its functions are chiefly opsonic. Clinical results appear to establish the success of treatment with salvarsan or its derivatives.

The laboratory confirmation of a diagnosis of malignant pustule presents no difficulties if material from the pustule is obtained at an early stage, as *B. anthracis* can easily be found

in microscopical preparations and can be isolated culturally. In the later stages, however, the bacilli may not be found either microscopically, culturally, or by animal inoculation, and it may not be possible to confirm the clinical diagnosis. In the case of respiratory or intestinal anthrax it is essential to isolate the bacillus, in the one case from the sputum and in the other from the fæces, and to prove its pathogenicity before making a diagnosis. It should be recollected that since *B. anthracis* does not spore in the body, it is wrong to heat the material examined, as by doing so any anthrax bacilli will be destroyed.

Where the case clinically resembles anthrax—especially where the patient's occupation has rendered him liable to infection, or where large Gram-positive bacilli are found microscopically in a suspicious lesion—serum should be administered without waiting for the final conclusions of the bacteriologist.

The isolation and identification of *B. anthracis* in hair or wool suspected of being infected is more difficult. The material should be washed in sterile saline. One half of the washings may be heated to 80° C. for ten minutes to kill non-sporing bacteria and the other half left unheated. From these two parts, used in various dilutions, a number of agar plates are made. Sub-cultures are made from suspected colonies and, if these are of bacteria microscopically resembling *B. anthracis*, the pathogenicity test may be applied. This is always necessary, as hair and wool may contain many bacteria closely resembling anthrax bacilli but which are not pathogenic.

### ***B. subtilis.***

This bacillus in some respects resembles the *B. anthracis*. It is a non-pathogenic organism, the chief interest of which is due to the fact that it is an exceedingly common contaminating organism in laboratory cultures. It is present in the soil and dust, in water and air and in fæces. It is commonly present in the straw and hay used for packing glass ware, which makes obvious the necessity of thorough sterilization before the

apparatus is used. The bacillus, which is a motile, sporing, Gram positive organism, measures from  $1.5$  to  $6\mu$  by  $0.8\mu$ . It occurs singly or in chains which are sometimes quite long. The spores are found in or near the centre of the bacilli.

It grows readily at air or body temperature, but only in the presence of air since it is one of the strictest of the aerobes, showing no evidence of growth in the absence of free oxygen. On agar it produces a dry, thin, tough, wrinkled film of a grey colour, which rapidly spreads over the surface of the medium. In broth a pellicle forms on the surface. Gelatin is liquefied, as also is coagulated serum to a slight extent, Carbohydrates are not attacked.

### ***B. mesentericus.***

This organism closely resembles *B. subtilis*, but is somewhat larger. It is fairly commonly found in the fæces, and its distribution in nature is similar to that of *B. subtilis*. The spores can withstand an exposure for ten minutes to a temperature of  $110^{\circ}$  C. This organism is responsible for the "ropiness" occasionally found in bread.

## CHAPTER XXVII

### *Corynebacterium diphtheriæ.*

Klebs and Löffler, 1883.

THE *C. diphtheriæ*, in preparations made from cultures on serum medium, is a straight or slightly curved bacillus. Its average size is  $3$  to  $5\mu$  by  $0.3$  to  $0.4\mu$ ; but great variations in size are met with, depending partly on the strain and partly on the culture medium used. It stains well with the aniline stains and is Gram positive, but usually strains which have been kept for some time in artificial culture resist decolorization less well than those lately isolated. The bacilli are rarely of uniform diameter throughout their length; most commonly one or both ends are somewhat wider than the central part, producing a "club" effect. On the other hand, certain individuals taper towards the ends, the central part being widest. Although some

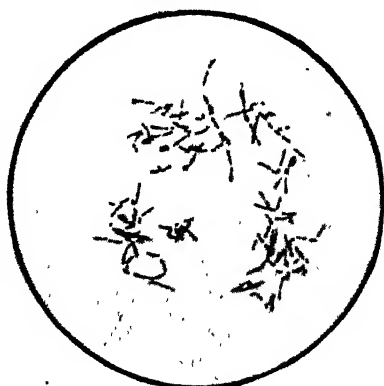


FIG. 51.—*C. DIPHTHERIÆ* FROM SERUM CULTURE ( $\times 800$ ).

strains of diphtheria bacilli stain uniformly, it is more common to find an arrangement of alternately darkly and lightly staining parts. This gives to the bacillus a characteristic beaded or striped appearance which, in extreme cases, may cause the organism to resemble, unless closely observed, a short chained streptococcus. This irregularity in staining is best observed with Löffler's alkaline methylene blue stain.

Frequently at the ends of the bacilli, especially if these are swollen, or more rarely, along the length of the organisms, intensely staining oval bodies, the polar bodies or metachromatic granules are seen. Special staining methods, such as that of Neisser, have been devised to show the polar bodies. By Neisser's method the granules are stained blue and the bodies of the bacilli brown. With an old, polychromatic methylene blue stain the granules appear of a reddish violet colour. In addition to these characteristics, certain other points which may assist in identifying the organism should be mentioned. There is, in a pure culture,

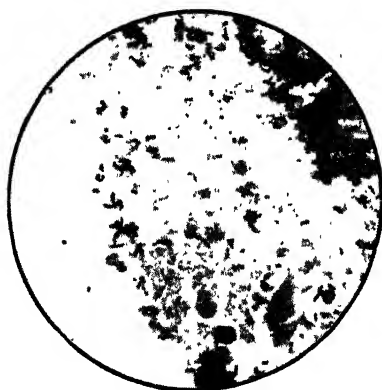


FIG. 52.—*C. DIPHTHERIÆ* IN FALSE MEMBRANE ( $\times 950$ ).

a marked lack of uniformity in size, morphology and staining peculiarities in the individual bacilli. Long and short forms, uniformly staining and granular bacilli are seen lying side by side. There is also a rather characteristic grouping of the bacilli which occur usually in pairs. The two organisms, however, are scarcely ever arranged end to

end, but most commonly close together and parallel, or with one end of one in contact with an end of the other, the two lying at an acute angle.

The above description applies chiefly to *C. diphtheriæ* as it is seen in culture on serum medium. Films prepared from agar cultures show bacilli which stain less typically and in which the development of granules is less marked. After 2 or 3 days' culture involution forms, chiefly large clubs, are plentiful. When films are made from a false membrane, the bacilli present a much less characteristic appearance. They are found in the membrane lying scattered irregularly, or in

small groups, chiefly towards the surface. They usually are somewhat shorter, stain more uniformly and show less tendency to produce "clubs" than do bacilli from cultures. Neisser's stain may fail to demonstrate polar bodies, or may succeed in only a small proportion of the bacilli.

The diphtheria bacillus is aerobic and facultatively anaerobic. It is easy to cultivate; but the best cultures are obtained on media containing blood or serum. Solidified serum, or one of the special media such as Löffler's, of which the basis is serum, is the medium of choice for the cultivation of *C. diphtheriæ*. On this, the colonies appear in less than twelve hours, that is more rapidly than the majority of other bacteria likely to occur about the throat. In consequence of this the result of culturing a throat swab on serum is to increase enormously the proportion of diphtheria bacilli to other organisms. The bacillus may grow but feebly on plain agar when freshly isolated, but in later cultures grows well. Primary cultures may always be obtained on blood agar or agar containing serum or ascitic fluid.

It has long been known that different strains of *C. diphtheriæ* present considerable differences in cultural characteristics. The work of McLeod and others has explained these differences as being due to the occurrence of three varieties of diphtheria bacilli—Gravis, Intermediate and Mitis. At first, for differentiation, reliance was placed on the form of colony produced on a special blood-tellurite medium, but this is now supplemented by other differential characteristics. The Gravis type, on the special medium, produces large, grey or grey-black, daisy head colonies: in broth it produces a heavy pellicle and granular deposit with the bulk of the fluid clear: it ferments starch and is not usually hæmolytic. The Intermediate type, on the special medium, produces very small, flat, black, granular colonies with central papillæ: in broth it causes a fine, dust-like deposit without clouding or surface growth; it does not ferment starch and is never hæmolytic. The bacilli are almost entirely of the barred form with poor development of metachromatic granules. The Mitis type, on the special medium, grows in colonies which



are black, glistening and smoothly convex; in broth it produces a uniform turbidity with or without a fine pellicle; it does not ferment starch and is hæmolytic. Several serological types of each of the three varieties occur.

All true diphtheria bacilli ferment glucose and the majority maltose, but none ferment saccharose.

Diphtheria bacilli have much greater powers of resistance than the majority of non-sporing bacteria. They may remain alive in dried false membrane for several months at room temperature, or for one hour at 98° C. When moist, however, an exposure to 60° C. kills them in a few minutes.

The natural disease in man affects chiefly the upper part of the respiratory tract, especially the fauces, the local lesion causing the formation of a "false membrane," which is composed of laminæ of fibrin, entangled in which are a few leucocytes or epithelial cells. The bacilli lie chiefly in the superficial (*i.e.* older) part of the false membrane. Underlying the false membrane, and more or less firmly attached to it, are the remains of the mucous membrane of the part, many of the cells of which have undergone necrosis. The local disease is frequently made more serious by the presence of other bacteria, particularly streptococci and staphylococci. A false membrane may be found in other parts of the body, such as the conjunctiva, vulva, vagina, and in wounds, but much more rarely than in the respiratory tract. In young children, a bloodstained discharge from the nostrils is frequently due to infection with the diphtheria bacillus.

The false membrane may cause death of the patient from obstruction to respiration, especially when situated in the larynx, but death is much more commonly due to the severe systemic disturbance caused by the disease. Although a few diphtheria bacilli may be found in the internal organs, such as the spleen, there is never, in this disease, a widespread infection or septicæmia. The remote effects are due entirely to the virulent toxin formed by the growth of the bacilli. To this toxin are to be ascribed the injuries to the kidneys, muscles and nerves, shown by the occurrence of albuminuria, myocarditis, and neuritis which were frequent

in the pre-antitoxin era and are still occasionally seen. The marked lowering in the blood pressure in the disease is probably to be attributed to the effects of the toxin on the adrenal glands.

In addition to man, the rabbit and guinea-pig, the ox, horse, cat, dog and small birds are susceptible to the effects of the bacilli or their toxin. The mouse and rat are relatively immune. These animals rarely, if ever, develop the disease naturally; but in the rabbit, for example, a false membrane similar to that in man may be produced by inoculation if the mucous membrane be previously injured by scarification. The subcutaneous inoculation of a guinea-pig with a sufficient amount of a virulent culture of bacilli produces death in 36 to 72 hours. The local lesion is a small greyish area of necrosis with some fibrinous exudation; surrounding this is an extensive zone of œdema with congestion and occasionally small hæmorrhages. The neighbouring lymph glands are swollen, œdematous and congested. The internal organs are usually congested. This is most marked in the adrenals, which are often hæmorrhagic. The epithelium of the tubules of the kidney and the cells of the liver exhibit cloudy swelling. Serous effusions may occur in the body cavities, and hæmorrhages in the serous membranes. These effects are due almost entirely to the toxin, since there is little invasion of the animal's body by the bacilli. In fact, the injection of toxin free from bacilli produces almost the same effects. If a smaller dose is administered, death may not occur or may be greatly delayed. A progressive paralysis, commencing in the hind legs, appears in from two to three weeks.

For the preparation of antitoxin the first essential is a potent toxin. There are a number of factors concerned in this, of which the most important is the strain of diphtheria bacillus used. No two strains have the same power of producing toxin, and the most satisfactory one, which is used to-day all over the world, was isolated by Park in New York, in 1895. It still produces toxin at least as powerful as when freshly isolated. The exact composition of the medium also requires great care. It should be alkaline and not readily yield to the acidity liable

to be produced by the growth of *C. diphtheriæ* when carbohydrates are present. It should contain a large proportion of peptone. The addition of sodium acetate and maltose (0.4 per cent.) to the medium improves the yield of toxin. Free access of oxygen is essential, for which reason bottles only partly filled and laid on their sides are used. For the same reason surface growth in the form of a pellicle is encouraged. There is always a certain period at which the amount of toxin present is at a maximum. This depends on the medium, size of bottle and other factors, and occurs in about 8 to 10 days. If this time is exceeded, the toxin becomes less powerful, for toxin is relatively unstable, especially if exposed to light and air. It retains its potency to a considerable extent for several months if kept in sealed tubes at a low temperature. Exposure to a temperature of 58° C. for a few hours or 100° C. for a few minutes completely destroys it, but, in the dry state, it is much less easily injured by heat. When it is believed that the amount of toxin is greatest, the broth is filtered through a porcelain filter. The toxic filtrate so obtained is usually spoken of as a "toxin," although the true toxin, which has never been isolated, is only one of the substances contained in it.

A typical toxic filtrate may have values similar to the following :—

M.L.D.	..	..	..	..	0.002 c.c.
M.R.D.	..	..	..	..	0.000002 c.c.
L <sub>+</sub> dose	..	..	..	..	0.180 c.c.
L <sub>r</sub> dose	..	..	..	..	0.175 c.c.
L <sub>t</sub> dose	..	..	..	..	0.155 c.c.

The M.L.D. is the smallest amount which kills a guinea-pig of 250 gms. weight in four days after subcutaneous inoculation.

The M.R.D. is the smallest amount which, injected intradermally into a guinea-pig, causes a red flush 5 mms. in diameter within thirty-six hours.

The L<sub>+</sub> dose is the amount of filtrate which, injected subcutaneously into a 250 gm. guinea-pig together with 1 unit of antitoxin, kills on the fourth day.

The  $L_r$  dose is the amount which, injected intradermally into a guinea-pig together with 1 unit of antitoxin, causes a red flush 5 mms. in diameter.

The  $L_t$  dose is the amount which, when mixed with 1 unit of antitoxin, flocculates more rapidly than does any other volume with the same amount of antitoxin.

One attack of diphtheria usually protects the patient against the disease for life as a result of the development of antitoxin. A considerable percentage of people, however, who have never suffered from diphtheria do not acquire it when exposed to infection. They may even become carriers of virulent diphtheria bacilli without any apparent ill effects. The bacilli are in their throats but the disease does not develop. The explanation is the presence in the serum of such persons of a small amount of antitoxin. Whether a person exposed to infection does or does not develop diphtheria depends very largely on whether or not antitoxin is present in the blood. This can be discovered by the Schick test. A small injection ( $\frac{1}{50}$  of a guinea-pig M.L.D. of toxin) is given intradermally in the arm. If there is no antitoxin, or only a very small amount, in the blood, a slight infiltration of the skin at the site of inoculation occurs in 24 to 36 hours. This is surrounded by a bright red areola a few centimetres in diameter. The red colour remains intense for more than a week, when it gradually passes off, but a brownish discoloration may remain for some weeks: this is a positive result. The amount of toxin used in the test was selected as likely to give a negative result in persons with more than  $\frac{1}{30}$  of a unit of antitoxin per c.c. in their sera, but recent work shows that negative results may be obtained when not more than  $\frac{1}{500}$  of a unit per c.c. is present, an amount which is not always sufficient to protect against the disease. It would seem desirable to increase the amount of toxin in the Schick test dose as, if this were done, diphtheria would occur among Schick negative persons even more rarely than it now does. Until the change is made even the faintest reaction should be considered as positive. In making the test, it is always necessary to inoculate the other arm with the same amount of "toxin" which has been heated to 75° C. to

destroy its toxin. This control reveals a pseudo-reaction which is non-specific, and is due either to the protein substances of the broth or to products of the growth of the organism, other than toxin. In a pseudo-reaction, both the site of the toxin and of the control injection exhibit a certain amount of infiltration and congestion. The Schick reaction is very useful, as it enables one to judge who are immune and whom it would be advisable to inoculate in order to confer on them either passive or active immunity.

The bacteriological diagnosis of diphtheria is usually fairly easy. Occasionally it may be made by the direct microscopical examination of material taken from the affected part with a swab. This procedure is not recommended except in an emergency and where the examination can be made by a highly experienced person. The bacilli are neither so plentiful nor so characteristic as in culture. The swab should be rubbed gently over the surface of a serum tube which should be incubated for 12 to 18 hours. Films are prepared by taking sweeps from this tube, and are stained by Löffler's alkaline methylene blue, by Gram's and by Neisser's methods. As a routine, the author prefers Löffler's stain, by which the morphology of the bacillus is most clearly seen; but some bacteriologists prefer Gram's stain. Occasionally some of the diphtheroid bacilli, such as Hofmann's bacillus, may cause confusion, for although a typical diphtheria bacillus is very different from a typical Hofmann's bacillus, atypical strains of the two may have a close resemblance to one another. It is always found, however, that, with increased experience, the number of cases in which any doubt exists greatly diminishes. In the case of a throat condition which is clinically suspicious, one is justified in reporting as a diphtheria bacillus any organism which has typical cultural and microscopical appearance. In the majority of cases the method outlined above is satisfactory but occasionally, especially when the swab was taken from a convalescent case before release, despite the selective action of solidified serum on the growth of *C. diphtheriæ*, the organism although present in the throat may not be found in films prepared from the serum culture.

For this reason it is strongly recommended that two cultures should always be made from swabs from the throat and nose—one on serum and the other on a medium containing tellurite, such as that of Horgan and Marshall or McLeod. After incubation over-night the serum culture should be examined microscopically and, if typical bacilli are found, the result should be reported as positive and both cultures discarded unless a virulence test is required, in which case the tellurite medium should be retained, as from it isolation is easier than from serum. If microscopic examination of the serum culture is negative, the tellurite medium should be incubated for a further 24 hours. Absence of black colonies warrants a negative report. If black colonies are present, films should be made and examined microscopically. If only cocci or *Oidium* are found, a negative report may be given, but if any bacilli are seen the colony containing them should be subcultured to serum from which, after 24 hours' incubation, films should be prepared and examined, typical morphology being accepted as presumptive proof of the presence of *C. diphtheriæ*.

When there is any doubt as to whether the organism reported as *C. diphtheriæ* on microscopical evidence is a virulent bacillus, or when the exclusion of a suspected carrier from school or employment is involved, something more than a microscopical identification is required. The final proof that an organism is a diphtheria bacillus depends on its power of toxin production. One fairly frequently meets with bacilli which microscopically and culturally are *C. diphtheriæ*, but which are avirulent and atoxic. Since it has never been established that a non-toxic *C. diphtheriæ* can be transformed into a toxin-producing bacillus, and since it is well known that a toxic strain retains its toxic properties even in artificial culture for many years, one is not justified in segregating as a carrier a person in whose throat or nose non-toxic bacilli resembling *C. diphtheriæ* are found. In the case of an epidemic, where a number of healthy persons are being examined in order to find carriers, those whose throats contain what may be called "morphological" diphtheria bacilli should be isolated for the few days necessary to establish whether or not their organisms are viru-

lent, but only those with virulent bacilli should be isolated until their throats are free from the bacilli. In order to prove that an organism is a virulent *C. diphtheriae* it must first be isolated in pure culture. This is most easily accomplished by the use of one of the tellurite media, such as that of Horgan and Marshall. The bacillus isolated must ferment glucose, producing acid only, fail to ferment saccharose and must be virulent for a guinea-pig. The best method of testing this is to inject the growth from a young culture on serum (one slope), suspended in saline, subcutaneously into each of two guinea-pigs, one of which has been protected by 250 units of antitoxin, injected the previous day. If the unprotected animal dies within five days, exhibiting the usual signs, and the other survives, the organism may be regarded as a true diphtheria bacillus. The passively immunized animal is required as certain corynebacteria which are not pathogenic for man but highly virulent for guinea-pigs may occasionally be found in the human throat and nose. If one of these is tested as described, both animals die, but neither shows typical post-mortem signs of diphtheria toxæmia. When several strains are to be tested for virulence the intradermal method may be used. A suspension of the organism is made which is just opalescent (about 500 millions per c.c.). The hair is removed from the flanks of two white guinea-pigs, one of which has previously been protected by an injection of antitoxin, and 0.2 c.c. of the suspension is injected into each intradermally. If the bacillus is virulent a red area of 1 to 2 cms. in diameter develops in the unprotected animal in 24 hours. The colour fades in three to four days leaving a necrotic area with a scab. The protected animal shows no lesion. By this method several cultures may be tested using the two guinea-pigs.

Since practically all Gravis and Intermediate varieties of diphtheria bacilli are toxigenic, it usually suffices to determine that the organism is a diphtheria bacillus (by morphology, fermentation of glucose and failure to ferment saccharose) and that it is either a Gravis variety (by colony form on McLeod's medium, growth in broth, and fermentation of starch) or an Intermediate variety (by colony form and growth in broth).

Only in exceptional cases is a virulence test of either variety essential. Many diphtheria bacilli of the *Mitis* variety are non-toxigenic and therefore, if a *Mitis* organism is isolated from a suspected carrier, a virulence test should be performed.

Diphtheria is most commonly spread by droplets from the mouths either of those with the disease or of carriers. The problem of the carrier is of great importance in the spread of diphtheria. Although in the majority of cases the bacilli disappear within a few weeks after the separation of the false membrane, in the remainder they may persist for a much longer time; in a few cases for six months or so. Many persons who have never suffered from diphtheria are carriers of the organism. In Dublin, in 1930, O'Meara found 5 per cent. of school-children to be carriers of "morphological" diphtheria bacilli and 2·3 per cent. carriers of virulent organisms. The carrier state is sometimes very intractable and may resist all the usual antiseptic measures. The most successful treatment is the removal of unhealthy tonsils and adenoids and, in the case of nasal carriers, surgical correction of any abnormalities and the use of mild alkaline douches or Dimol snuff.

In all cases where carriers are being looked for, nasal swabs in addition to throat swabs should be examined.

On account of the difficulty of finding and rendering harmless all carriers, immunization, either locally as in an institution such as a school or more wide-spread, as in a city, has been recommended. By means of the Schick reaction the immune may be differentiated from the susceptible, only the latter requiring attention. Passive immunity may be conferred on those who are exposed to infection by the injection of 500 units of antitoxin. This immunity is of but brief duration, a few weeks at most. A much more lasting immunity may be established by the subcutaneous injection of a neutral mixture of toxin and antitoxin but, since any preparation containing toxin is potentially dangerous and since toxoid is quite safe and antigenically at least as active as toxin, only some preparation containing toxoid should be employed.

Three prophylactics are now commonly employed—formol toxoid (F.T.), alum precipitated toxoid (A.P.T.) and toxoid-



antitoxin floccules (T.A.F.). Formol toxoid (anatoxine) is prepared by treating toxin with 0.2-0.4 per cent. formalin for 4 to 6 weeks at 37° C. A.P.T. is obtained by adding alum to toxoid, the aluminium hydroxide which is precipitated adsorbing the toxoid which it carries down. The precipitate is centrifuged and washed to free it, as completely as possible, from non-specific material. T.A.F. is the insoluble precipitate formed by the combination of toxoid and antitoxin when the two are present in optimal proportions. It also is purified by centrifuging and washing. Three intramuscular injections, each of 1.0 c.c., of F.T. or T.A.F. at intervals of two or three weeks will make more than 90 per cent. of Schick positive individuals Schick negative within six weeks of the last injection. A single injection of 1.0 c.c. of A.P.T. may sometimes change the Schick condition, but if this prophylactic is used it is advisable to give at least two injections at an interval of two or three weeks. The first of these may be of 0.1 c.c. and the second of 0.5 c.c. The first (detector dose) will show if the person is likely to react badly to a larger dose. If, with it, reaction occurs, T.A.F. should be used subsequently. Even with A.P.T., three injections (0.1, 0.5 and 0.5 c.c.) are preferable to two. The choice as between the three depends largely on the severity of the reactions which may follow injection. These reactions are due chiefly to the sensitivity of some persons to the products of growth of the diphtheria bacillus other than toxin. The age of the individual treated is an important factor. Children under the age of six are very rarely sensitive to these substances but, with increasing age, the proportion of sensitive persons increases. For children under this age, therefore, either F.T. or A.P.T. may be used with little fear of bad reactions. Older children or adults should not be treated with F.T. or A.P.T. until, by the use of a small (detector) dose, it has been determined that they are not unduly sensitive. For the immunization of reactors, T.A.F. should be used as it is the least likely of all the prophylactics to give rise to reactions. If the detector dose produces no reaction, either F.T. or A.P.T. may be employed with safety. Since the majority of children under

the age of eight are Schick positive, these may all be immunized without Schick test. Older children and adults (especially doctors and nurses) should be Schick tested and positive reactors immunized. In all cases, about two months after the completion of the course of immunizing injections, the Schick test should be performed to determine if immunity has been produced. A small percentage will be found to be Schick positive and will require further treatment. If this "posterior" Schick test be omitted, these positive reactors may contract diphtheria and bring immunization into disrepute.

Every case which is clinically suspicious of diphtheria should at once receive antitoxin treatment *without waiting for the bacteriologist's report*: 5,000 to 50,000 units, according to the severity of the case, should be administered, and this may be repeated until there is definite improvement. Cases of sore throat which are not clinically diphtheria, but in which C. diphtheriæ is present, should also receive antitoxin. Enormous doses (up to 200,000 units) are given in severe cases by some practitioners of great experience, and it is claimed that, by such treatment, mortality is much lower, paralysis of less frequent occurrence and convalescence shorter than with the more usual doses.

In connection with the administration of antitoxic serum in diphtheria, as in other diseases, the route is a matter of considerable importance. In very severe cases and in cases seen at a late stage, the route of choice is the intravenous one as the antitoxin is introduced into the circulation without delay. In other cases the intramuscular route is to be preferred to the subcutaneous. When serum is administered intramuscularly it reaches its maximum concentration in the blood in 24 hours, whereas, by the subcutaneous route, maximum concentration in the blood is attained in 72 hours.

There can be no doubt as to the efficacy of the antitoxin treatment of diphtheria if it is applied sufficiently early, but the delay of even one day greatly increases the risk of a fatal termination of the disease. If antitoxin is not administered within the first week it will probably do little to lessen the

mortality, although, in the survivors, late post-diphtheritic paralysis will be less common in those who receive serum.

Within the past few years the severity and fatality of diphtheria have greatly increased in many areas. This is apparently due to an increased prevalence of Gravis and Intermediate types of diphtheria bacilli, infections with which are more serious and less responsive to antitoxin than are those with the Mitis type. Since all types produce the same toxin, it is only possible to advise the use of antitoxin

as early as possible and in large doses.

The term "diphtheroid bacillus" is rather loosely applied to any Gram positive, non-motile, non-sporing bacillus resembling more or less closely the diphtheria bacillus in microscopic appearance. As has been pointed out before, many bacilli appear to be identical with *C. diphtheriæ* in microscopic and

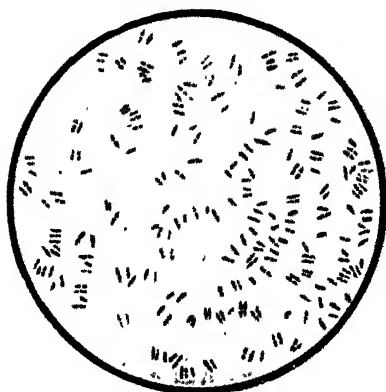


FIG. 53.—*C. HOFMANNI* FROM CULTURE ON SERUM ( $\times 800$ ).

cultural characteristics, save that they do not produce toxin. These should be called "non-toxic diphtheria bacilli," and the term "diphtheroid" only applied to bacilli which, while not producing toxin, may be differentiated in other ways. Some, for example, do not ferment glucose, and since all true diphtheria bacilli produce acid from this sugar, this fact is sufficient to distinguish them from the true bacilli of diphtheria.

The pseudo-diphtheria bacillus of Hofmann, which is probably completely devoid of pathogenicity, derives its importance from its frequent occurrence in the throat and nose and the possibility of its being confused with *C. diphtheriæ*. It is shorter and thicker than this organism, has tapering ends, and has not usually a beaded or granular appearance. Polar

staining is absent. It stains deeply and evenly with methylene blue save for the presence of a single, central, unstained septum. More rarely two unstained bars may occur. In evolution forms, a common feature in old cultures of *C. diphtheriæ*, do not occur in Hofmann's bacillus. The colonies are cream or white in colour, and are larger and less transparent than those of *C. diphtheriæ*. It does not ferment any sugar and is non-pathogenic to the guinea-pig.

The Xerosis bacillus, which is frequently found in the conjunctiva, both normal and diseased, resembles *C. diphtheriæ* very closely in microscopic appearance. It is not pathogenic for animals and produces acid in media containing glucose, maltose, and saccharose.

Other diphtheroid bacilli are found in the respiratory, intestinal and urinary tracts, in wounds and in other localities. None of these appears to be primarily pathogenic, but they may act as secondary invaders after a lesion has been caused by other organisms.

The *C. acnes* is usually classed with the diphtheroids. It is a short, thick bacillus, frequently club-shaped, which grows only under anaerobic or partial anaerobic conditions. For its culture we have found that broth to which is added 1 per cent. of glucose and 1 per cent. of oleic acid, the whole being covered with a layer of oil, is a satisfactory medium. It also grows well in semi-solid glucose agar stabs. It occurs in acne pustules and comedones either alone or together with a staphylococcus.

## CHAPTER XXVIII

### COLIFORM BACILLI

**Bact. coli.** (*B. coli.*) *Escherichia coli.*

Escherich, 1886.

BACT. coli is one of the most widely distributed of all bacteria. It is found in the intestines of man and of very many animals as a harmless or, more probably, beneficial saprophyte. It is of great importance to the bacteriologist for many reasons. Outside the intestinal canal, it may produce various morbid conditions in the body. Inside the intestine it is usually non-injurious; elsewhere in the body it is pathogenic and is to be classed with the other pyogenic bacteria. It is almost constantly found in human and animal fæces, and for this reason must be familiar to the bacteriologist who has frequently to examine such material for the presence of the specific organisms of typhoid fever, dysentery or other diseases, which more or less closely resemble it. Finally, its prevalence in excreta gives it importance as an "indicator organism" in the examination of water, milk, or other food materials. If Bact. coli is present in these, they have been exposed, at some period, to fæcal contamination.

The average size of Bact. coli is from 1 to  $5\mu$  by  $0.5\mu$ ; but no great reliance should be placed on its size, since this is very variable, depending on the strain and culture medium used. It may sometimes be so short as to appear almost coccal, at other times filamentous forms 10 to  $15\mu$  in length may be found, especially in the urine. It possesses 6 to 8 flagella arranged round it and is motile, but usually not very actively so: some strains are so sluggish that motility can be detected in them only with great difficulty. Each bacillus is

generally free, but pairs, arranged end to end, or short chains of three or four bacilli are sometimes seen. It stains readily and is Gram negative.

*Bact. coli* grows on all ordinary media and is aerobic and facultatively anaerobic. The colonies on agar plates are of large size and are circular in shape; their colour is white, greyish, or brownish, and they are rather opaque, with moist glistening surface. On gelatin their appearance is very similar to that on agar; no liquefaction is produced. In broth *Bact. coli* causes a uniform turbidity with a slight tendency to sedimentation and pellicle formation after some days. Indol is produced in cultures in broth and in peptone water. *Bact. coli* is an active fermenter of sugars, glucosides, and alcohols, with the production of carbon dioxide and hydrogen in approximately equal proportions. In glucose broth a high degree of acidity is developed. This is permanent and so the methyl-red test is positive. The most important substances fermented are lactose, glucose, maltose, mannitol, and dulcitol. Saccharose is not fermented. Acid and clot are produced in milk. In "shake" cultures in agar or gelatin containing glucose, the medium is broken up, owing to the evolution of gas from the sugar present. Typical *Bact. coli* does not give the Voges-Proskauer reaction and fails to grow in Koser's medium. On blood agar some strains are hæmolytic, but the majority are non-hæmolytic.

While confined to the intestine, *Bact. coli* acts merely as a saprophyte, but in other parts of the body it may take on a definitely pathogenic rôle. Where the integrity of the mucous membrane of the intestine, which normally keeps it within its proper bounds, is interfered with, as in typhoid fever and dysentery, or the whole intestinal wall is damaged, as in strangulated hernia or volvulus, the *Bact. coli* of the intestine can penetrate into neighbouring tissues and cause suppuration. The healthy intestinal wall is a sure protection, but if damaged is no longer a safe barrier. Appendicitis, peritonitis, and perirectal abscesses are sometimes caused by *Bact. coli*, either alone or associated with other organisms. If the flow of bile is arrested the organism may penetrate into the gall-

bladder, setting up a cholecystitis. It may infect the urinary system, particularly in the female or in the male after catheterization, causing cystitis and pyelitis. It is one of the causes of puerperal sepsis. Gunshot wounds are often infected with the organism. As a sequel to abdominal operations *Bact. coli*, alone or with other bacteria, may lead to the production of pelvic or subphrenic abscesses, empyema or abscess



FIG. 54.—*BACT. COLI* IN DEPOSIT OF URINE FROM CASE OF CYSTITIS ( $\times 950$ ).

in the lung. *Bact. coli* septicæmia is uncommon and rarely occurs save as a terminal phenomenon of diabetes and other chronic diseases; but local conditions, which are almost certainly due to infection by the blood stream, may be seen in the occasionally noticed pneumonia, pleurisy, osteomyelitis, and meningitis caused by the bacillus. *Bact. coli* is definitely a pyogenic organism, and even injections of dead bacilli may cause local abscesses.

The rabbit and guinea-pig are susceptible to infection by *Bact. coli*, subcutaneous inoculation causing a local abscess, while the intraperitoneal route frequently gives rise to a fatal peritonitis. Intravenous injection commonly produces a fatal septicæmia.

When *Bact. coli* has taken on a definitely pathogenic rôle the patient's serum is frequently found to agglutinate the organism responsible for the condition. Normally a person's serum does not agglutinate a saprophytic, intestinal *Bact. coli*.

Therapeutic anti-sera are not effective in the treatment of *Bact. coli* infections, owing chiefly, in all probability, to the very large number of types of this organism which exist. In

fact the description "Bact. coli" is commonly used to include many different organisms, which have little in common save that they are inhabitants of the intestine and ferment lactose. For the treatment of Bact. coli infections, vaccines, which must be autogenous and prepared from the organism present in the diseased part and not from the intestine, are often of great service. This is particularly the case with cystitis or pyelitis, in which conditions, however, a rather prolonged course of injections, attaining a dose of considerable size, may be necessary. It should be noted that Bact. coli vaccines are rather toxic and the initial dose should be small.

It is probable that the newer drugs (mandelic acid and the sulphanilamides) will entirely replace vaccines in the treatment of Bact. coli infections of the urinary tract.

For the isolation of Bact. coli from fæces, urine, water or other material, one of the special media, of which MacConkey's agar may be taken as a type, may be used. Lactose-fermenting bacteria produce red colonies. These should be subcultured in the various sugar and other media mentioned above in order to determine, as exactly as possible, whether the organism is to be classed as a true Bact. coli or as one of the somewhat similar organisms.

**Bact. aerogenes.** (*B. lactis aerogenes*) *Aerobacter aerogenes*.  
Escherich, 1885.

This is a short, non-motile Gram-negative bacillus which grows luxuriantly on agar, producing thick, white, moist, viscid colonies. It is a vigorous fermenter of carbohydrates, producing acid and gas from lactose, glucose, maltose, mannitol, and saccharose. Dulcitol is occasionally fermented. In the fermentation of glucose the degree of acidity developed is less than with Bact. coli and also is less permanent, so giving a negative methyl-red test. The volume of carbon dioxide produced from glucose is double that of the hydrogen. The bacillus does not liquefy gelatin; indol is not usually produced; the Voges-Proskauer reaction is positive. The bacillus is capable of growing freely in medium such as that of Koser in which the only source of carbon is a citrate. Some



strains of *Bact. aerogenes* are capsulated, which may give rise to difficulty in distinguishing them from *Bact. pneumoniae*.

The organism appears to be chiefly a saprophyte, being found in soil, water, and grain; it is one of the common causes of the souring of milk. It is not uncommonly found in small numbers in human faeces and may occasionally be pathogenic for man, causing cystitis or other types of inflammation; it has been found in the blood stream in septicæmia in a few cases.

The chief importance of this bacillus lies in the fact that it is commonly found in water supplies, particularly in tropical countries and, as it ferments lactose, it may be confused with *Bact. coli*. Since it is widely distributed in nature, its presence in water has not the significance attached to *Bact. coli*, which is predominately faecal in origin.

Fairly closely related to *Bact. aerogenes* is *Bact. cloacæ* (*Aerobacter cloacæ*), which, however, causes liquefaction of gelatin.

Other bacteria which, in their characteristics, are intermediate between *Bact. coli* and *Bact. aerogenes* are commonly found in water and give rise to difficulty in assessing its sanitary quality. Among these is the organism formerly known as *Bact. coli communior*, which differs from *Bact. coli* chiefly in fermenting saccharose. The sanitary importance of *Bact. coli*, *Bact. aerogenes*, and the intermediates was considered in Chapter X.

## CHAPTER XXIX

**Bact. typhosum.** (*B. typhosus*, *Bact. typhi*) *Eberthella typhosa*.  
Eberth and Gaffky, 1884.

THE typhoid bacillus possesses no morphological characteristics sufficiently definite to distinguish it from the other members of the group of Gram-negative intestinal bacilli. It exhibits considerable pleomorphism and in cultures occasional long filamentous forms may be observed. It possesses about 12 flagella, arranged peripherally, and is, in general, very actively motile.

This organism grows well, though less luxuriantly than *Bact. coli*, on all the ordinary culture media. On agar the colonies, which are thin and film-like, are at first very transparent, appearing a bluish-grey colour by transmitted light and grey or white by reflected

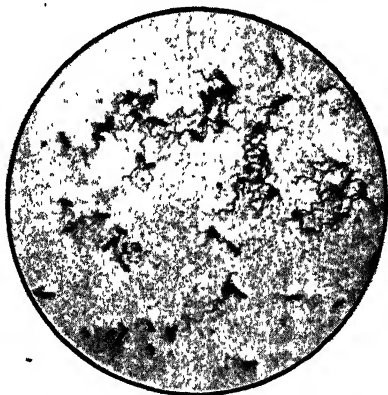


FIG. 55.—BACT. TYPHOSUM SHOWING FLAGELLA ( $\times 950$ ).

light. The colonies of recently isolated bacilli are smooth and circular in shape, but old laboratory strains which have undergone the S→R degradation may produce colonies which are somewhat rough with coarsely crenated margins. On MacConkey's agar the colonies, which are smaller than those of *Bact. coli*, are almost colourless, and highly reflecting. (See Plate III.) In broth the organism causes a uniform turbidity. It grows slowly on gelatin and does not liquefy this medium.

The fermentative powers of the typhoid bacillus are less marked than those of *Bact. coli*. Acid, but no gas, is produced in media containing mannitol, glucose, and maltose. Lactose, saccharose, and dulcitol are not fermented. In milk slight acidity is caused, followed by alkalinity. No indol is produced by the growth of this organism in peptone water. *Bact. typhosum* does not give a positive Voges-Proskauer reaction.

The terms "typhoid" and "enteric" fevers were used synonymously before the bacteriology of the conditions was



FIG. 56.—*BACT. TYPHOSUM* FROM AGAR CULTURE ( $\times 750$ ).

known. Since two names are unnecessary for one disease, it is recommended that typhoid fever should be used only for the disease due to *Bact. typhosum*, while enteric be applied to any of this group of diseases, whether due to the *Bact. typhosum* or to one of the paratyphoid bacilli. On clinical grounds the diagnosis of enteric fever in the patient would be made.

Later, as the result of laboratory investigation, a more definite diagnosis, either of typhoid, paratyphoid A, paratyphoid B or paratyphoid C fever would be established. In the same way the "enteric group" of bacilli would include *Bact. typhosum*, *Bact. paratyphosum* A, B and C.

*Bact. typhosum* is the cause of typhoid fever in man. In this disease the most marked effects are produced in the intestine, particularly in the lymphoid tissue—Peyer's patches and solitary follicles—of the small intestine. It must not be supposed that the disease is exclusively one of the intestine; it is a general disease with marked local changes in this part of the body. In the early stages the lymphoid tissue

exhibits an acute inflammation with congestion and emigration of leucocytes, chiefly mononuclears: small hæmorrhages may also be found. At this time typhoid bacilli are present, most commonly in small clusters, in the inflamed tissues. Later necrosis occurs and the dead tissue is sloughed off, leaving ulcers. Death may be occasioned by hæmorrhage from these ulcers, or by perforation of the wall of the intestine leading to peritonitis. While the lymphoid tissue of the intestine is undergoing these changes, the mesenteric glands also become enlarged and show a similar acute inflammation. Necrosis is rarer in these, but it occasionally occurs. The spleen is enlarged, fairly firm and congested and of a reddish pink colour. In it large numbers of typhoid bacilli may be found, usually in clusters between the cells. Similar collections of the bacilli are, more rarely, found in the liver. A very hæmorrhagic type of pneumonia also occurs with *Bact. typhosum* as the causal organism. In addition to its more general rôle, the *Bact. typhosum* must be regarded as a pyogenic bacterium. Abscess formation—subcutaneous, in the parotid gland, in the kidney and elsewhere—may occur either as a complication of typhoid fever or, not infrequently, years after recovery from the disease. In some cases only the *Bact. typhosum* may be found in these abscesses; sometimes this organism may be associated with others, *Bact. coli*, streptococci, and staphylococci. The *Bact. typhosum* is of almost constant occurrence in the gall-bladder during the disease and there may set up a catarrhal or purulent cholecystitis. These widespread effects of the *Bact. typhosum* are not to be wondered at, since the bacillus is found in the blood stream in the first week of the disease in almost every case. In fact, blood culture is the earliest and most certain method of diagnosing the disease. During the course of the disease there is a mononuclear leucocytosis in the blood.

Freshly isolated typhoid bacilli are pathogenic for the ordinary laboratory animals, the mouse being most commonly used, but in no animal (excepting the anthropoid apes) is it possible to produce a disease resembling the typhoid fever of man. Subcutaneous, intraperitoneal, or intravenous inocula-

tion produces commonly a short acute illness, with fever and loss of appetite: death may occur in 24 to 72 hours. Intravenous inoculation of rabbits frequently produces a septicæmia and the bacilli may be isolated, after death, from the gall-bladder. The cause of death in experimental animals is chiefly the toxin (endotoxin) of the typhoid bacillus which is, apparently, associated with O type antigens.

The disease is conveyed most commonly from man to man by water, milk, or other food which has become infected from someone who has, or has had, the disease. The bacilli are present in the fæces in every case and in the urine in about 25 per cent. of the cases of the fever. Normally they cease to be excreted a few weeks after convalescence, but in from 2 to 5 per cent. of cases they persist in the fæces for much longer periods, for months or for years. Such persons are known as "fæcal carriers." The reservoir of the bacilli in fæcal carriers, who are usually women, is the gall-bladder alone or the gall-bladder and liver. Urinary carriers are less common than fæcal carriers, but are a much greater danger to the public owing to the greater possibility of the dissemination of bacilli from the urine than from the fæces. Fæcal carriers have been known to excrete the bacilli for as long as thirty years after an attack of the disease.

It is frequently necessary to look for a carrier among a number of persons. The serum of a carrier usually agglutinates the typhoid bacillus, but this may be true also of a person who has suffered from the disease or been vaccinated against it. The O type of agglutination is of greater value than the H, and it has been asserted that Vi agglutinins rapidly disappear from the serum after convalescence unless the carrier condition is established, in which case they persist. A history of having had the disease, or of recurring pain or tenderness in the region of the gall-bladder is occasionally helpful. It is, however, always necessary to isolate the organism from the fæces or urine. The method of doing so will be described later. It should be mentioned that the carrier state may be intermittent; no typhoid bacilli may be detected by daily examination for days or weeks, and then suddenly they may be found in

large numbers. They may persist for a few days and then another latent period occurs. For these reasons great patience and perseverance are often necessary in the search for a typhoid carrier. The cure of the carrier condition may be difficult, or impossible; drugs, operations such as removal of the gall-bladder, and vaccines have been tried with limited success. A carrier, once found, should be kept under observation, so that he or she may not be able to contaminate a water supply or to handle food.

One attack of typhoid fever probably protects against the disease for life. The serum of a patient, in the later stages of the disease, has acquired considerable anti-bacterial properties. It is not strongly antitoxic but is bactericidal and bacteriolytic; it acquires the property of immobilizing and agglutinating the bacilli and also, to a lesser extent, has increased opsonic power. An animal, such as a rabbit, repeatedly inoculated first with dead and later with living bacilli, acquires a similar immunity, and in its serum identical properties may be found.

Until the increased employment of blood culture for the bacteriological diagnosis of typhoid fever, the favourite method was the Widal test. This test, if properly performed with suitable materials, is still of great value where blood cultures cannot be made. The suspensions of typhoid bacilli used should be of both H and O and possibly also of Vi types.

Agglutination reactions are of the greatest assistance in the identification of a suspected bacillus as *Bact. typhosum*. A rabbit is immunized with a known typhoid bacillus and its serum is collected when its titre is  $\frac{1}{2000}$  or upwards. If this serum agglutinates the suspected organism at nearly the same dilution as its titre for the homologous organism, the presumption is that the new bacillus is a true *Bact. typhosum*.

Subcutaneous inoculation of killed bacilli, especially when these are of a smooth and highly virulent strain, confers on man a very real protection against typhoid fever and if, despite this vaccination, the disease does develop, the mortality is very much less in those vaccinated. The immunity is less permanent than that due to the natural disease, but appears to be

of useful degree for one or two years after the administration of the vaccine. Every army in the European War had a wonderful demonstration of the efficacy of anti-typhoid inoculation. The sickness rate from enteric fevers in the British army during that war amounted to about 2 per thousand, while in the South African war it was 105 per thousand. The case mortality in South Africa was 13.9 per cent. as compared with 5.9 per cent. in the European War. Usually 500 million bacilli constitute the first dose and 1,000 millions, ten days later, the second. It is now the routine practice to combine with the typhoid vaccine, vaccines of the two paratyphoids (T.A.B.). Small doses of vaccine have been used for the treatment of typhoid fever, without any great success.

Many efforts have been made to prepare useful antisera for the treatment of typhoid fever but, until recently, these have not been successful. Now, however, as a result of the work of Felix and others, the outlook is more hopeful and it is believed that, if an animal is immunized with bacilli rich in both O (toxic) and Vi (virulence) antigens, its serum may be therapeutically active in virtue of the antitoxin and bacterial antibodies which it contains.

### Diagnosis of Typhoid Fever.

**1. Blood Culture.**—By far the most certain and easy method is by blood culture. Preferably bile salt broth should be used, the bile salts or bile itself serving to prevent clotting of the blood and, much more important, inhibiting the bactericidal properties of the patient's serum and leucocytes. About 5 c.cs. of blood, taken from a vein at the elbow with a sterile syringe, should be added to 100 or 150 c.cs. of the broth in the special bottle. After incubation over-night a loopful is spread on the surface of a solid medium and the next day suspected colonies are subcultured and tested as described below. Blood culture is positive in about 90 per cent. of cases in the first week, 75 per cent. in the second, 50 per cent. in the third and 25 per cent. in the fourth week, after which it is rarely positive.

**2. Isolation of the Bacilli from the Fæces.**—This is more difficult than isolation from the blood owing to the large numbers of *Bact. coli* present, but is greatly facilitated by the use of newer media.

The following method can be recommended as a result of personal experience. Two tubes of tetrathionate broth are inoculated with the fæces, one heavily (0.5 gm.) and the other lightly (1 loopful). One loopful from each tube is, at once, spread on a plate of Wilson and Blair's medium. Both tubes and both plates are incubated. Next day a loopful from each tube of tetrathionate broth is spread on a plate of MacConkey's medium; these are incubated. On the following day the MacConkey plates which have been incubated for 24 hours and the Wilson and Blair plates which have been incubated for 48 hours are examined. Pale colonies from the MacConkey plates and black colonies from the Wilson and Blair plates, if present, are individually subcultured on to agar slopes for further investigation.

With these methods, a positive result may be expected in about 75 per cent. of cases in the first week, the percentage of successes increasing to about 85 per cent. in the second week and 95 per cent. in the third.

**3. Isolation from the Urine.**—A fresh specimen should be obtained under aseptic conditions and several loopfuls spread over the surface of the medium favoured. The isolation of the bacilli is then continued as in the case of fæces. The urine contains, at some period, typhoid bacilli in about 25 per cent. of cases. Occasionally the number of bacilli may be large, one million or more per cubic centimetre. The bacilli are never present in the urine before the third week of the disease.

**4. Isolation of the Bacilli from Other Parts of the Body.**—Before blood cultures were practised the bacilli were occasionally isolated by plating a small portion of spleen juice, obtained during life by aspiration with a syringe. Owing to the great congestion of the spleen this is a dangerous and unjustifiable procedure. Cultures from the spleen and from the gall-bladder should, however, be made at post-mortem



examinations. Occasionally the bacilli may be obtained in culture from the rose spots, sputum, pus or vomit.

When a suspected organism has been obtained in pure culture, in any of these ways, it is identified by testing its fermentative actions on "sugars." If it agrees with the classical description, is motile and does not produce indol, an agglutination test is put up with a suspension of the organism and an anti-typhoid serum of known titre. If the serum agglutinates the organism to nearly full titre (say  $\frac{1}{1500}$  with a serum of  $\frac{1}{5000}$  titre) the bacillus may then be reported as *Bact. typhosum*. It is of importance to note that occasionally a true typhoid bacillus, when freshly isolated from the body, is relatively, or even totally, inagglutinable by H type agglutinins but not by those of O type unless it is tested in the living state.

**5. Widal Reaction.**—Failing, from any cause, to obtain blood cultures, diagnosis is most easily made by Widal's reaction. This has the great advantages that only a small amount of blood is needed, and that the specimen can be sent by post to a laboratory at a distance from the patient. The macroscopic method should be used. The reaction (H and O types) is rarely positive before the beginning of the second week; it increases in intensity during the course of the disease and reaches a maximum about the end of the third week. Standard agglutination (H type) at a dilution of  $\frac{1}{50}$  is almost diagnostic, so long as it is certain that the person has not had typhoid fever previously and has not been inoculated against the disease. In the case of inoculated persons, in whom the reaction may remain positive for years, it may be necessary to repeat the test once or twice. An increase in the H titre of a patient's serum is taken, by some, to indicate the probability that the disease is typhoid fever. With a suspension of O type bacilli a titre of  $\frac{1}{100}$  is rare except in those suffering from typhoid. It is usual for the serum of a typhoid patient to contain both H and O agglutinins, but in some cases only one of these is present. It is now accepted that O type agglutination is of greater diagnostic significance than H type.

It is claimed that, while positive agglutination reactions with H and O type suspensions are merely presumptive evidence of the disease, tests with Vi suspensions are never positive except during the actual disease or the carrier condition. During the disease the Vi titre rises sharply at the end of the first week, attaining its maximum between the tenth and the twentieth day, after which it falls rapidly. At its highest, the titre may be from  $\frac{1}{25}$  to  $\frac{1}{200}$ .

## CHAPTER XXX

### SALMONELLA BACILLI CAUSING ENTERIC FEVER

#### **Bact. paratyphosum A.**

(*B. paratyphosus A*), *Salmonella paratyphi*.

Brion and Kayser, 1902.

#### **Bact. paratyphosum B.**

(*B. paratyphosus B*), *Salmonella schottmülleri*.

Schottmüller, 1900.

#### **Bact. paratyphosum C.**

(*B. paratyphosus C*), *Salmonella hirschfeldii*.

Hirschfeld, 1919.

THE *Salmonella* group (or genus) includes many bacilli with similar cultural and serological characteristics but with very dissimilar pathogenic properties.

In a certain proportion of cases, enteric fever is due not to *Bact. typhosum* but to *Bact. paratyphosum A*, *B* or *C*, or possibly to one of the other members of the *Salmonella* group. Of these, *A* occurs chiefly in tropical countries and America, *B* in the tropics, and *C* in eastern Europe. All except *B* are rare in the British Islands.

The diseases produced by these organisms closely resemble typhoid fever in pathology and in symptoms but are almost always of a much milder type, the mortality being considerably lower.

Morphologically, the three organisms present no distinguishing characteristics. All are actively motile. In their fermentative reactions they are almost identical. Acid and gas are produced by their growth in media containing glucose, maltose, and mannitol, while lactose and saccharose are not fermented. *Bact. paratyphosum C* ferments dulcitol actively, but *A* and *B* may fail to ferment this carbohydrate or may ferment it slowly. Xylose is not fermented by *A*, may or may not be fermented by *B*, and is always fermented by *C*.

In general, *Bact. paratyphosum* A is a less active gas producer than are the other two.

Owing to their close resemblance, it is preferable to rely chiefly on serological rather than on cultural characteristics for the identification of the organisms. All are members of the *Salmonella* group but possess few antigenic characteristics in common. *Bact. paratyphosum* A is monophasic and its serological differentiation from the others is simple. *Bact. paratyphosum* B and C are both diphasic but their differentiation presents little difficulty. It is, however, much harder to distinguish them from some other members of the *Salmonella* group.

The methods of diagnosis of the paratyphoid fevers are similar to those of typhoid fever. Blood culture is the method of choice, but faeces culture is also useful. When agglutination tests are used, it is important to note that, in these diseases, the titres of the patient's sera are usually lower than in typhoid fever. Both H and O suspension should be used. Vi antigen has not been detected in *Bact. paratyphosum* A or B, but appears to be present in *Bact. paratyphosum* C.

Immunity can be produced in man against the paratyphoid fevers by vaccines, just as in the case of typhoid fever. The first dose contains 250 millions of each bacillus and the second 500 millions. T.A.B. vaccine contains typhoid bacilli and paratyphoid A and B bacilli. Paratyphoid C bacilli should be added in countries where this organism occurs.

## SALMONELLA BACILLI CAUSING FOOD-POISONING

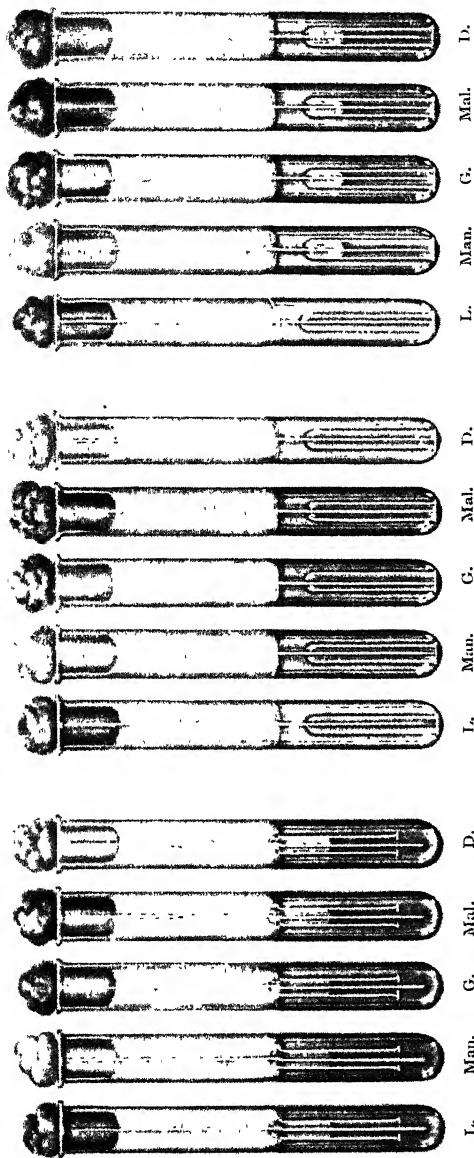
In addition to the members of the *Salmonella* group causing enteric fever in man, there are very many which cause disease in lower animals. Many human outbreaks of bacterial food-poisoning are due to chance invasions of the human body by some of these which are also pathogenic for man. The most commonly isolated have been *Bact. typhi-murium* (*B. ærtrycke*), *Bact. enteritidis* (Gaertner's bacillus), and *Bact. cholerae-suis* (*B. suipestifer*). More recently isolated organisms

of the group are given specific names derived from the places where they were first isolated—*Bact. newport*, *Bact. derby*, *Bact. reading*, *Bact. dublin*, etc. The organisms are very similar in their cultural characteristics to one another and to *Bact. paratyphosum B.* Serological methods are required for their differentiation and, owing to the sharing of antigenic constituents, this is often very difficult, requiring elaborate antigen analysis by the absorption of agglutinin technique. In view of the close relationship of the members of the group to one another, the differentiation of so many species scarcely appears to be warranted.

In man the symptoms of bacterial food-poisoning are those of an acute and severe gastro-enteritis. The mucous membrane of the stomach and intestine is intensely inflamed and sometimes hæmorrhagic: occasionally a septicæmia may be present.

The method of infection is not always easily discovered. Some of the organisms cause enteritis with septicæmia in animals, the flesh of which is used for human food. This is true of the *Bact. enteritidis* which causes disease in cattle. It is possible, therefore, that the flesh of a sick animal might cause disease in man. In a number of cases infection has occurred through eating eggs, especially those of the duck. Both *Bact. typhi-murium* and *Bact. enteritidis* have been found in such eggs, into which they were apparently introduced before the eggs were laid by a duck infected with the bacilli. In the majority of cases, however, some other method of infection must have occurred. Since rats are commonly infected by *Bact. enteritidis* and mice by *Bact. typhi-murium*, severe enteritis being induced, there can be little doubt that infection of food is commonly due to these animals, the bacilli being conveyed to the food either directly or indirectly by flies or dust. Human carriers have very rarely been discovered. Frequently the responsible food has been re-heated meat, hashes and the like, or such articles as meat pies. In the majority of these cases the organism was probably introduced into the food during some stage of its preparation and had sufficient time to develop before its consumption. Milk and milk pro-

# PLATE IV.



*B. coli.*

*B. typhosus.*

*B. paratyphosus B.*

## Sugar Reactions of some of the Coli-typhoid Group.

L. = lactose ; Man. = mannitol ; G. = glucose ; Mal. = maltose ; D. = ducitol.



ducts such as ice-cream have, occasionally, conveyed the infection. Not only may disease in man be caused by the living bacillus, in which case symptoms do not develop for about twenty-four hours after eating the food, but also by the toxins produced by the growth of the bacillus. These toxins are exceedingly heat-resisting and may even withstand boiling for 10 to 15 minutes, so that re-cooked food may, in its second heating, have been held at a temperature sufficient to kill the bacilli but not to destroy the toxin. Symptoms due to the toxin, without living bacilli, are similar to those caused by the bacilli, but occur within an hour or two of partaking of the food.

Where living bacilli have been ingested, a diagnosis may be made by isolating the organism from the vomit or fæces by methods similar to those used for isolating *Bact. typhosum*. The food-poisoning bacilli are not usually present in the blood. The serum of a recovered case may contain antibodies which cause the agglutination of the causative organism.

In addition to the *Salmonella* bacilli, very many different types of bacteria can grow in food and there produce toxic substances which cause bacterial food-poisoning. Among these are some of the dysentery bacilli, staphylococci, *Cl. botulinum*, and probably others.



## CHAPTER XXXI

### THE DYSENTERY BACILLI

DYSENTERY is the clinical name for a group of diseases with completely different ætiologies. It may be due to the effects of a protozoon, the *Entamœba histolytica*, or of several types of bacilli. It is only the latter which will be considered here.

The dysentery bacilli may be divided into two main groups, the non-mannitol-fermenting bacilli and those which ferment mannitol. Both are morphologically indistinguishable from *Bact. typhosum*; they are, however, non-motile, although their very active Brownian movement may be mistaken for true motility.

**Bact. shigæ.** (*B. dysenteriae* (*Shiga*)), *Shigella dysenteriae*.  
Shiga, 1898.

The chief non-mannitol-fermenting dysentery bacillus is *Bact. shigæ*. This bacillus is culturally very like *B. typhosus*, the colonies on agar being rather small and transparent. It ferments glucose, producing acid but no gas, and does not ferment any of the other sugars. It does not produce indol in peptone water.

In man the disease due to this bacillus is a local one of the intestine, chiefly of the large intestine, and the blood stream or other parts of the body are rarely invaded. Certain nervous symptoms are, however, caused by the toxin of the organism. The earliest pathological change produced is a catarrhal inflammation of the mucous membrane. Later, the surface may be covered with false membrane, while the mucous membrane itself is intensely congested. There is a general fibrinous œdema and cell infiltration of the wall of the intestine,

which is considerably thickened. Necrosis and sloughing of the mucous membrane occurs, producing the characteristic ulcers. The stools in the acute stages are fluid, containing a considerable amount of mucus and pus usually accompanied by blood. The cause of death is the intense toxæmia rather than the local damage to the intestine, but this latter may allow other bacteria, streptococci for example, to penetrate into the tissues and give rise to septicæmia. The mortality varies in different epidemics but frequently exceeds 20 per cent.

Infection is conveyed from patients or carriers by the fæces. Urinary carriers, so important in typhoid, do not exist in dysentery. The carrier state may last for years, and the carrier frequently suffers from recurrent symptoms owing to chronic intestinal lesions.

*Bact. shigæ* is pathogenic for most animals, chiefly owing to its toxicity, a definite exotoxin being produced. The bacilli themselves are also toxic.

The intravenous administration of toxin or bacilli to a rabbit causes a rapid fall in temperature and violent diarrhœa, at first watery and later bloody: if death is delayed paralysis may occur. It seems probable that the symptoms in man are due chiefly to the action of the toxins and not to the bacilli themselves. It is possible to immunize horses or other large animals, and the sera of these are strongly antitoxic. They are of great benefit in the treatment of the disease in man but must be given in large doses, preferably intravenously or intramuscularly.

Diagnosis is made by the isolation of the causative organism from the stools. Blood cultures are useless, as the bacilli are not usually present in the blood. The earlier in the disease the stools are examined, the greater are the chances of isolating the bacillus. Another important detail is that the cultures should be made as soon as possible after the passage of the stool. If it is impossible to examine a specimen of fæces fresh, it may be kept in a condition suitable for culture by the addition of 2 parts of a 30-per-cent. solution of glycerol in saline to 1 part of fæces. A small flake of mucus, preferably bloody, should be thoroughly washed in two or three changes

of sterile saline and spread over the surface of a plate of MacConkey's or other medium. After 24 hours, suspected colonies may be sub-cultured and put on sugars. Neither Wilson and Blair's medium nor tetrathionate broth should be used as they inhibit the growth of dysentery bacilli. The final identification may be made if the isolated bacillus is agglutinated by an anti-Shiga serum, approximately to its full titre.

Agglutination of a known *Bact. shigæ* by the patient's serum is occasionally useful for diagnosis. Agglutinins appear on the second or third day of the disease, but a diagnosis can rarely be made by this method before the seventh or eighth day. It is usually stated that if agglutination occurs in a dilution of  $\frac{1}{50}$ , a positive diagnosis may be made. The author found, however, in an epidemic, that by adopting this standard about one-half of the cases would be pronounced negative. A great deal depends on the strain of bacillus used, as different strains vary greatly in agglutinability. Since the bacillus is non-motile, only O type agglutination occurs.

### Schmitz's Bacillus.

(*Bact. ambiguum*), *Shigella ambigua*.

Schmitz, 1917.

This is a less common cause of dysentery than Shiga's bacillus which, in many respects, it resembles. Glucose is the only commonly employed carbohydrate fermented by it. Schmitz's bacillus produces indol in broth. The two organisms display some antigenic similarity but can be distinguished from one another by direct agglutination.

**Bact. flexneri.** (*B. dysenteriae* (Flexner)), *Shigella paradysenteriae*.

Flexner, 1900.

The mannitol-fermenting dysentery bacilli are best classed together as Flexner dysentery bacilli. They grow rather more luxuriantly than does Shiga's bacillus; all produce indol and all ferment glucose and mannitol; some, in addition, ferment maltose; gas is not produced from any carbohydrate.

Antigenically the Flexner dysentery bacilli are very complex. A serum prepared against one agglutinates the majority of the other bacilli of the group. Four main types, V, W, X and Z are recognized and it seems probable that each of these has, possibly in varying proportions, a number of common group antigens and, in addition, a specific type antigen.

These bacilli generally produce a very much milder disease in man than does Shiga's bacillus; they are also very much less toxic for laboratory animals. As is the case with infections due to Shiga's bacillus, the disease is spread by the faeces of patients and carriers.

The methods of diagnosis are the same as for the *Bact. shigæ*. Since normal human serum may agglutinate Flexner bacilli to a considerable extent, no conclusions should be drawn from agglutinations occurring at a lower dilution than  $\frac{1}{100}$ .

It is commonly found that from the faeces of cases of clinical dysentery, organisms, resembling in morphology and cultural characteristics the Flexner type of bacillus, are isolated. Such organisms, however, are not agglutinated by agglutinating sera prepared against known dysentery bacilli, and their ætiological connection with the disease has not been established.

The Newcastle bacillus which appears to be serologically distinct from the other dysentery bacilli is also distinguished from them by the fact that it produces a small amount of gas from glucose and dulcitol. It does not produce indol.

### **Bact. sonnei.**

(*B. dysenteriae* (Sonne)), *Shigella paradysenteriae*, var. *Sonne*,  
Sonne, 1915.

Another type of dysentery bacillus, the Sonne bacillus, appears to be responsible for dysentery, usually of a mild type, occurring in various parts of the world. It has also caused at least one outbreak of food-poisoning in which the incubation period was about 12 hours and death occurred within 24 hours of the consumption of the food. This organism, which is

non-motile and Gram negative, produces on MacConkey agar large, irregular, crenated colonies with papillæ. The organisms in these, after some days, ferment lactose and so the papillæ become pink. It is distinguished from the other dysentery bacilli by the fact that it slowly ferments lactose (usually in from 5 to 10 days). It also ferments glucose, maltose and mannitol and usually saccharose, gas not being produced. It does not produce indol and does not liquefy gelatin. Serologically it is distinct from the other dysentery bacilli and is very slightly, if at all, agglutinated by an anti-Flexner serum.

THE DYSENTERY BACILLI

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	Motility.	Lactose.	Mannitol.	Glucose.	Maltose.	Dulcitol.	Milk.	Indol.
<i>Bact. coli</i> .. ..	+	A G	A G	A G	A G	A G	A C	+
<i>Bact. typhosum</i> .. ..	+	—	A	A	A	—	A	—
<i>Bact. paratyphosum A</i> ..	+	—	A G	A G	A G	A G or —	A	—
<i>Bact. paratyphosum B</i> ..	+	—	A G	A G	A G	A G	A-alk.	—
<i>Bact. enteritidis (Gaertner)</i>	+	—	A G	A G	A G	A G	A-alk.	—
<i>Bact typhi-murium</i> ..	+	—	A G	A G	A G	A G	A-alk.	—
<i>Bact. shigæ</i> .. ..	—	—	—	A	—	—	—	—
<i>Bact. flexneri</i> ...	—	—	A	A	A or —	—	—	+
<i>Proteus morgani</i> .. ..	+	—	—	A G	—	—	A-alk.	+

\* A = acid only.

A C = acid and clot.

+ = motile or indol produced.

A G = acid and gas.

A-alk. = acid followed by alkaline reaction.

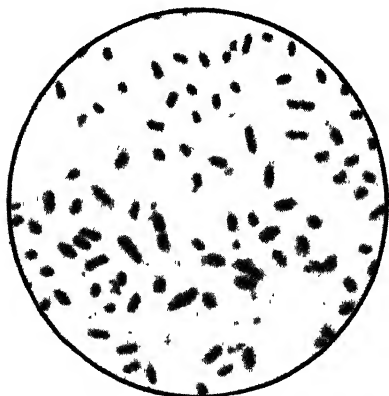
— = non-motile, no indol produced, or no change in reaction.

## CHAPTER XXXII

### SOME OTHER BACILLI

**Bact. pneumoniae** (*B. mucosus capsulatus*, *Pneumobacillus*  
of Friedländer). *Klebsiella pneumoniae*.  
Friedländer, 1882.

BACT. pneumoniae is a pleomorphic organism which may vary in length from 0.6 to 4 $\mu$  and in thickness from 0.5 to 1.2 $\mu$ . In the body it is most commonly seen in almost coccal form, and was, in fact, originally described as a coccus and for some time was confused with the pneumococcus. It occurs singly or, very commonly, in pairs; much more rarely in short chains. It is non-motile and Gram negative. As seen in secretions



or in early cultures it is supplied with a well-marked capsule. No spores are produced.

It grows readily on ordinary media, best under aerobic conditions, and either at the temperature of the body or at 20° C. On agar it produces large, grey, or white, raised colonies, which appear moist or semi-fluid and tend to be confluent. These

FIG. 57.—BACT. PNEUMONIAE SHOWING CAPSULES (X 750).

are found to be viscid or mucoid when touched with the platinum wire. On an agar slope the growth, which is luxuriant, tends to gravitate to the bottom of the tube. On blood agar a wide zone of hæmolysis is produced around

each colony. In a gelatin stab a white line of growth is formed, and on the surface the organisms grow in an elevated bead, giving to the whole the appearance of a nail with a large head. In broth there is turbidity with the development of a slimy sediment and of a pellicle. Indol is only occasionally produced. Probably owing to the occurrence of various strains the fermentative activities of the bacillus are varied. Most usually glucose, mannitol, maltose and saccharose are acted upon, acid and gas being produced; lactose may or may not be fermented. The Voges-Proskauer reaction is usually negative and the methyl-red test positive. Most strains of *Bact. pneumoniae* grow on Koser's medium.

The organism is fairly commonly found about the respiratory tract, and frequently appears to play no pathogenic rôle. It may, however, in a small percentage of cases, be the cause of pneumonia of a very severe type. It has occasionally been the cause of abscess formation in the respiratory tract, of suppuration in the nasal and frontal sinuses and in the antrum of Highmore. Cases of otitis media, empyema, pericarditis, meningitis and septicæmia have been produced by it, and it is commonly associated with both acute and chronic bronchitis.

Subcutaneous injection of the organism into the guinea-pig and mouse usually causes a local abscess, but occasionally a fatal septicæmia develops. Intraperitoneal injection leads to peritonitis and septicæmia. The rabbit is more resistant against the bacillus and injections rarely produce more than local abscesses.

A bacillus either identical with or closely related to Friedländer's pneumobacillus is found in the rare disease rhinoscleroma—a chronic granulomatous condition of the mucous membrane of the nose or upper part of the respiratory tract. The organism is found both between the cells and also, in considerable numbers, in large swollen cells, the cells of Mikulicz. In ozæna—a nasal catarrh characterized by the development of a foetid odour—a similar bacillus is almost constantly found, and is believed by many to be causative.

The majority of the members of this group fall into one of three serological types, but there are probably further types



not yet differentiated. As with the pneumococci, type specificity depends on the presence of a carbohydrate substance in the capsule.

***Pseudomonas pyocyanea.*** (*B. pyocyaneus.*)

*Pseudomonas æruginosa.*

Schröter, 1872.

*Ps. pyocyanea*, which measures from 1·5 to 5 $\mu$  by 0·3 to 0·6 $\mu$ , is found most commonly in pairs, but single bacilli and short chains also occur frequently. The organism is actively motile, has no capsule and does not form spores. It is Gram negative.

It grows readily on ordinary media at body temperature under aerobic conditions, but growth can also take place at the temperature of the air and in the absence of oxygen. The outstanding feature of cultures of the organism is the development in the medium of a green or greenish colour. This is due to the presence of two pigments, produced by the growth of the bacillus, of which one, called pyocyanin, is of a blue colour and the other, fluorescin, is greenish-yellow and fluorescent. For the production of the colour the presence of oxygen is essential. After some time the colour darkens, and the medium may become dark reddish-brown or almost black. On agar a greyish, moist, glistening growth with a metallic sheen is produced, which shows very little if any of the characteristic colour on the surface, since the pigments diffuse through the medium, giving to it the greenish-blue colour. Gelatin is rapidly liquefied. In broth, which becomes only slightly coloured, a turbidity is produced with the formation of a pellicle and a flocculent deposit. In cultures enzymes which liquefy or dissolve gelatin, casein, coagulated serum, and fibrin, are produced and an enzyme, pyocyanase, which has the power of dissolving other bacteria.

*Ps. pyocyanea* is widely distributed, being found in the æces and on the skin, apparently as a harmless saprophyte, as well as in water and soil. It is a common secondary invader of diseased tissues, and is met with in the upper respiratory tract and in dirty wounds, in which it may play an important

part in delaying healing and in lowering the general health of the patient. Its pathogenic powers are comparatively slight, and so the organism is found as the primary cause of disease chiefly in badly nourished children or in adults whose general health is poor. In these it has produced abscesses in various parts of the body, otitis media, peritonitis, pericarditis, empyema and broncho-pneumonia. It appears to have been responsible for some cases of gastro-enteritis in children and has been found in the blood stream in septicæmia. The lesions caused by it have characteristically a discharge which is green or blue in colour.

Of the laboratory animals the guinea-pig is the most susceptible. Intraperitoneal inoculation results in peritonitis, and the death of the animal in from 24 to 72 hours. Subcutaneous inoculation may cause only a local abscess with considerable oedema and necrosis, but most usually death follows. In the rabbit a local abscess is the most common result of subcutaneous injection, but occasionally the condition becomes chronic, the animal exhibits wasting, and death follows after some time.

The pathogenic effects of the organism are due both to a thermostable exotoxin and to an endotoxin. An antitoxin can be prepared which is capable of neutralizing the exotoxin. The pigments are not markedly toxic for animals.

### ***Proteus vulgaris.* (*B. proteus.*)**

Hauser, 1885.

*Pr. vulgaris* is usually from 0.6 to 0.8 $\mu$  in thickness, but of very varied length from almost coccal forms of 1 $\mu$  to long filaments. It is actively motile, has no capsule, and does not form a spore. It stains readily but is Gram negative.

*Pr. vulgaris* grows readily on ordinary media under aerobic and anaerobic conditions, both at air and body temperatures. On agar it produces colonies which rapidly spread ("swarm") in an irregular manner over the surface of the plate, forming a thin, flat, shining, moist, grey-white layer. Gelatin is usually liquefied under aerobic conditions only, but in some strains

the ability to liquefy it has been lost. Milk is coagulated and the clot subsequently digested. In broth there is a heavy turbidity, together with pellicle formation and a mucoid sediment: indol is usually produced. Acid and gas are produced in glucose and saccharose and, by some strains, in maltose, but not in lactose or mannitol. The organism is usually definitely proteolytic, and is capable of digesting egg albumin, fibrin, and coagulated serum. It produces  $H_2S$ . It does not give a positive Voges-Proskauer reaction.

*Pr. vulgaris* is commonly found in soil, and in water rich in organic matter, and is frequently present in the fæces of man and animals. It is one of the organisms of putrefaction, and cultures have an unpleasant odour, similar to that obtained in putrefying material.

It has not a high degree of pathogenicity, but may be the sole cause of pus formation in man. It is most commonly found alone in cystitis and pyelitis and has occasionally been responsible for otitis media, pleurisy or abscesses. It is frequently present, with other organisms, in contaminated wounds, and appears to be one of the causes of delayed healing in them. In some epidemics of summer diarrhoea in infants *Pr. vulgaris* appears to be the chief organism present in the intestine. It has been stated that food contaminated with *Pr. vulgaris* may cause food-poisoning, but it appears improbable that this can often be the case, owing to the unpleasant odour and taste of food so affected.

The organism has a low degree of pathogenicity for laboratory animals, but injections of large amounts of a culture may cause death. The same result will, however, follow massive injections of other bacteria, commonly regarded as non-pathogenic.

**Proteus morgani.** (*Morgan's No. 1 Bacillus.*)

*Salmonella morgani.*

Morgan, 1911.

This is a Gram negative, motile bacillus which ferments glucose with gas production but none of the other carbohydrates. It produces indol freely. On soft agar at air temperature it "swarms" in the same way as *Pr. vulgaris*.

It has been isolated from the fæces of infants suffering from summer diarrhoea and may be one of the causes, but not the only one, of that condition. It is frequently present in healthy stools. It causes diarrhoea and death in young rabbits when administered by the mouth.

**Bact. alkaligenes.** (*Alcaligenes fæcalis*.)

This is a short, motile, Gram negative, non-sporing bacillus which is found singly, in pairs or chains. It does not liquefy gelatin, produce indol or ferment any carbohydrate. It is a normal inhabitant of the intestine and may be recognised on MacConkey plates prepared from fæces by the yellowish zone surrounding the colonies, which is due to alkali production. It has but feeble pathogenic power.

#### GRAM POSITIVE INTESTINAL BACILLI

A considerable number of bacilli, which may be grouped together under the term "lactic acid bacilli" and which constitute the genus *Lactobacillus*, are found in the human intestine. They are all Gram positive, non-motile, non-sporing bacilli which ferment carbohydrates, usually without gas production. The most important member of the group is *Lactobacillus acidophilus*. This organism, which is found in large numbers in the fæces of breast-fed infants, usually measures from 4 to 5 $\mu$  in length, but shorter forms also occur. It is rather stout and occurs singly and in chains. On agar it produces minute, thin, feathery, transparent colonies. It leads to the production of acid and clot in milk; it produces acid without gas in lactose, glucose, maltose and saccharose. It is commonly used to sour milk for therapeutic use. When sufficient milk culture is consumed, preferably with added lactose, the fæcal flora is usually transformed into one consisting chiefly of *L. acidophilus*, proteolytic bacteria being inhibited by the acid reaction resulting from the fermentation of lactose. Smaller numbers of the bacilli are found in the fæces of adults living on ordinary diet and they can be isolated by cultivating the fæces in an acid medium (broth to which has been added 1 per cent. of acetic acid, for example).

Although the organism can withstand a degree of acidity fatal to most bacteria, it grows best at about the neutral point. Aciduric would, therefore, be a more suitable description than acidophilic. Döderlein's bacillus which, by the fermentation of glycogen, is largely responsible for the acidity of the vagina, is probably only a variety of *L. acidophilus*.

*Lactobacillus bulgaricus*, a smaller organism than *L. acidophilus*, is most commonly found in long chains. It ferments lactose and glucose but not maltose. It was formerly employed, to a considerable extent, in sour milk therapy but less successfully than *L. acidophilus* as only the bacilli introduced are found in the faeces, the organism failing to grow in the intestine.

The Boas-Oppler bacillus (*Lactobacillus boas-oppleri*) may be found in the stomach in cases where the hydrochloric acid is reduced in amount, as in gastric carcinoma, and particularly where there is pyloric obstruction. Its characteristics are, in general, similar to those of *L. acidophilus*, although it is usually both longer and stouter than that bacillus. It ferments lactose and glucose and grows best under micro-aerophilic conditions, that is where the amount of oxygen has been reduced below that present in the atmosphere.

*L. odontolyticus* has been isolated from carious teeth, and it is stated that the acids produced by this organism from carbohydrates are an important factor in the causation of dental caries. It closely resembles *L. acidophilus*.

A considerable number of other members of the genus *Lactobacillus* have been isolated from milk and other carbohydrate-containing materials in a state of fermentation, but none are of medical interest.

*L. bifidus* differs from the above in being an obligatory anaerobe on first isolation: later it may grow in air. It is a Gram positive, non-motile, non-sporing bacillus of very variable length. The ends are frequently swollen and sometimes bifurcated. It ferments lactose, glucose, maltose, and saccharose. It is found in the faeces of infants, especially those which are breast fed, and usually exceeds in numbers the *L. acidophilus* also present.

## CHAPTER XXXIII

### **Vibrio cholerae.** *Vibrio comma.*

Koch, 1884.

THE cholera vibrio measures from  $1.5$  to  $2\mu$  by about  $0.5\mu$ . As seen, usually in enormous numbers, in the dejecta from cases of cholera, it is definitely curved, and, from its resemblance to a comma, the name "Comma bacillus" was given to it. The curve, however, is not flat, but is in two planes. Frequently the organisms occur in pairs, giving either a C or, more commonly, S shape. Only rarely are long spiral chains found in the intestinal contents. Where a film is made from a small flake of mucus in the watery discharge the majority of the vibrios appear to lie with their long axes parallel, this appearance being described as resembling "fish in a stream." In cultures—particularly of old laboratory strains—the curved shape is almost or completely lost, and the organism seems to resemble one of the members of the coli-typhoid group. Old cultures may show large, badly stained involution forms which are commonly clubbed or coccal in shape. In fluid media long spiral chains of vibrios are of frequent occurrence. The cholera vibrio stains with the ordinary stains less easily than the

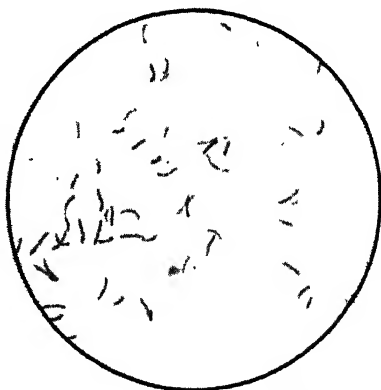


FIG. 58.—*V. CHOLERA* FROM AGAR CULTURE ( $\times 950$ ).

majority of other bacteria and is Gram negative. The vibrio is actively motile, owing to the presence of a single terminal flagellum. It has no capsule and does not form spores.

The organism grows best aerobically: under strictly anaerobic conditions very poor growth is obtained. It is not very restricted as regards the type of medium used, but alkalinity is essential; it will grow readily on media sufficiently alkaline to prevent the development of the majority of other pathogenic bacteria. It flourishes best at body temperature, but rapid growth also takes place at room temperature.

On agar the colonies are round, thin, very transparent and almost colourless. In old cultures, however, the agar frequently becomes brownish. Gelatin is rapidly liquefied, as is also coagulated serum. In broth or peptone water of pH 8.0 to 9.0 a heavy turbidity is produced, and on the surface a pellicle is formed, owing to the organism's preference for a liberal supply of oxygen. Indol and nitrites are rapidly produced. The "cholera red reaction"—the production of a pink colour on the addition of a mineral acid to a peptone water culture of the cholera vibrio—is due to the presence of both indol and nitrites in the culture. Acid but no gas is produced by the organism's growth in media containing glucose, mannitol, maltose, and saccharose, but fermentation may be slow. The majority of true cholera vibrios are non-hæmolytic.

The resistance of the vibrio is but slight. Against heat it resembles other non-sporing bacteria; but it is very susceptible to drying, being thus killed in a few hours. It can, however, resist low temperatures well, and is still viable after freezing for several days. Its life in stools is not long, rarely more than one or two weeks. In the water of wells or rivers it does not usually survive for more than a fortnight. It has been found to live for as long as two months in damp soil.

Cholera in man is an acute disease in which, in the great majority of cases, the causative organisms are confined strictly to the intestine, although the body as a whole suffers from the most intense toxæmia, as shown by the subnormal temperature

and the profound collapse and prostration. The lower half of the small intestine is the region most affected. The wall is congested and, owing to the penetration of the cholera vibrios between and beneath the epithelial cells, the latter are loosened and desquamated. The content of the gut is watery, and in it float flakes composed of masses of separated epithelial cells and of mucus: the common term "rice water stool" is a fairly good description of the patient's motion. The intense diarrhoea, which is accompanied by severe cramps, dehydrates the body to such an extent that the blood is concentrated and the urine is very scanty. Where the disease is more chronic, there may be extensive necrosis of the mucous membrane and the formation of a false membrane on the surface. The vibrios are found in enormous numbers in the fluid contents of the intestine, up to one thousand million per cubic centimetre having been observed. They penetrate beneath the epithelial cells, but not deeper, and although they have been found in the spleen, lungs and urine, their occurrence in such situations is exceptional. They are found, however, with considerable frequency in the gall-bladder.

Among the outstanding features of the disease are: (1) its short incubation period, usually one or two days; (2) its rapidity of development, death frequently occurring within twelve hours of the onset of the disease; and (3) the intense toxæmia. The first two of these are explained by the rapid rate of growth of the cholera vibrio, whether within the body or in vitro. The third feature is due to the intense toxicity of the organism itself. Most workers have found that filtrates of young broth cultures are but very slightly toxic; that is, an extracellular toxin is either absent or occurs only to a small extent. The most important toxin is the endotoxin contained within the bodies of the vibrios. To it are due the toxic effects experienced by man during the disease and by animals injected with the organism, living or dead. The liberation of the toxin in the bodies of man or of animals is due largely to the bacteriolytic action of serum on the vibrios. The toxin is found in filtrates of old broth cultures owing to



autolysis, and can be liberated artificially by grinding up cultures when frozen.

An enormous amount of work has been done to elucidate the problems of cholera experimentally. That the cholera vibrio is the cause of the disease has been proved by several experiments on man, some intentional and some accidental, in which the swallowing of pure cultures has occasioned the disease. Others, however, have escaped, and it seems probable that in order to give the vibrios a start in the intestine, some abnormal condition of the gastro-intestinal tract is either essential or at least of assistance. In this connection it is of interest that healthy carriers—contacts who have not had the disease but who excrete the vibrios in their stools—are known, and that they may, possibly as a result of some dietary indiscretion, acquire the actual disease. It is remarkable how often the layman attributes cholera to indulgence in over-ripe fruit.

No animal, under natural conditions, suffers from cholera, but artificial infections are possible in many. Feeding experiments are usually unsuccessful, except in the case of suckling rabbits, in which the smearing of the mother's teats with a culture is sufficient to produce a condition fairly closely resembling the human disease. In guinea-pigs it was found necessary to neutralize the acidity of the gastric juice by sodium bicarbonate and to paralyse peristalsis by opium before the administration of cultures into the stomach: under these conditions death occurred in a few hours with great prostration. The small intestine was distended with colourless fluid, containing epithelial flakes and large numbers of vibrios. Intravenous inoculation of rabbits usually leads to an infection of the intestine with the characteristic signs of the disease, and often also of the gall-bladder. Intraperitoneal injection in the guinea-pig produces a peritoneal effusion, subnormal temperature and collapse. Similar effects may be produced by injections of killed instead of living cultures.

As a result either of the natural disease in man or of the repeated injections of dead followed by living vibrios in animals a considerable degree of immunity is established and the serum

of the man or animal is found to contain antibodies to the organism. It is very slightly, if at all, antitoxic, as can be well shown by the intraperitoneal injection into a guinea-pig of a culture of cholera vibrios followed by immune serum. Owing to the rapid lysis of the vibrios and the consequent liberation of their endotoxin the animal dies more rapidly than another which has received a corresponding dose of culture without serum. Pfeiffer's work on bacteriolysis was done largely with this organism.

The use of anti-cholera serum in the treatment of the disease has proved disappointing. Vaccines have been found useful prophylactically; either a living vaccine of an attenuated culture or an ordinary killed vaccine being employed.

During the course of cholera enormous numbers of vibrios are excreted in the fæces and the disease is spread by the contamination of water supplies or food with such excreta. The vitality of the organism outside the body is not great, but the existence of the disease is maintained by carriers. The carrier state does not usually last for more than a fortnight after convalescence, and its existence for two months is uncommon, chronic carriers being exceedingly rare. Healthy carriers are a great danger both to themselves and to others. In suspicious waters vibrios, indistinguishable from the cholera vibrio except that they are not agglutinated by an anti-cholera serum, are commonly found. Their relationship to the disease, if any, is as yet uncertain.

Many other vibrios, more or less closely related to the cholera vibrio, are known. Of these the most important is the "El Tor vibrio," which was isolated from fæces during an epidemic of a dysenteric condition. It resembles the true cholera vibrio very closely in microscopical and cultural characteristics, but is actively hæmolytic and produces an extracellular toxin. It is agglutinated and dissolved by anti-cholera serum. Most observers now regard it as a modified cholera vibrio. Metchnikoff's vibrio, derived from an epidemic among fowl, causes in pigeons, on injection, an acute septicæmia. It is not agglutinated or dissolved by anti-cholera serum. The other vibrios, such as those of Finkler and Prior

and of Deneke, can all be distinguished with ease by their serological differences.

The diagnosis of cholera in man can frequently be made by direct microscopical examination of the fluid stools, both stained and unstained: the occurrence of large numbers of typical vibrios is almost conclusive. It may be possible to perform an agglutination test on the vibrios in the stool itself, using anti-cholera serum. In the absence of an epidemic or in an investigation of carriers, it is necessary to isolate the organism. This can be done most easily by adding a small portion of the stool to alkaline peptone water (pH 8.4). After six hours' incubation a loopful, removed from the surface, will usually show large numbers of vibrios; but if necessary a second inoculation may be made from the first, and in the case of carriers even a third may be required. The very rapid surface growth of cholera vibrios secures their enrichment and they can then be isolated by plating a loopful from the surface on alkaline agar or a special alkaline blood-agar, Dieudonné's medium. Direct plating of the stool may be used when the number of vibrios present is large. The final proof that the vibrio isolated in pure culture is that of cholera, is by finding that it is agglutinated by anti-cholera serum, acting in a dilution not far removed from its end point.

A serum acting on the O type antigens must be used. Three such antigens have been described in *V. cholerae*. H type antigens are useless for the identification of the cholera vibrio.

Owing to the acuteness of the disease, agglutination of cholera vibrios by the patient's serum is not very useful, but it may be necessary to resort to this procedure in order to make a diagnosis in a convalescent. Normal serum may cause agglutination at a dilution of  $\frac{1}{20}$ , and it is not usually safe to base a diagnosis on agglutination unless the serum acts in a dilution of  $\frac{1}{100}$  or more: it is then fairly reliable.

## CHAPTER XXXIV

### ***Pasteurella pestis.*** (*B. pestis.*)

Yersin and Kitasato, 1894.

PAST. pestis is found in its most characteristic form in material taken from early plague lesions in man or animals. It is short and thick, with rounded ends, and may frequently appear almost coccical in outline. Its average size is from 1 to 2 $\mu$  by 0.5 to 1.0 $\mu$ , but under certain conditions much larger forms are also found. It occurs in the body either singly or in pairs, short chains being exceptional. Young cultures on solid or in fluid medium present a very similar appearance, but there is a tendency for the organism to become more definitely bacillary when grown on solid medium. In cultures a few quite long thread-like forms are of frequent occurrence. Old lesions, old cultures, or young cultures on "Salt agar" (agar containing about 3 per cent. sodium chloride) show many involution forms, large, faintly staining, ovoid, globular, or club shaped, somewhat suggestive of yeasts.

The organism stains readily with ordinary stains, but is Gram negative. When freshly isolated from the body, a characteristic feature of the bacillus is the occurrence of polar staining. The central part of the bacillus is unstained, or but faintly stained, while each pole takes the stain intensely. By continued artificial culture this appearance is, to a considerable extent, lost. Past. pestis possesses a capsule which, however, is not always easily demonstrable. It is non-motile and forms no spores.

The plague bacillus grows best under aerobic conditions: in the complete absence of oxygen growth is either absent or very slight. The optimum temperature of cultivation is from 30° to 35° C., but growth occurs quite satisfactorily at

body temperature. It grows readily on any of the ordinary laboratory media, but the first cultures made from the body may require an enriched medium, such as blood agar. On agar the colonies are small, translucent, with granular centres

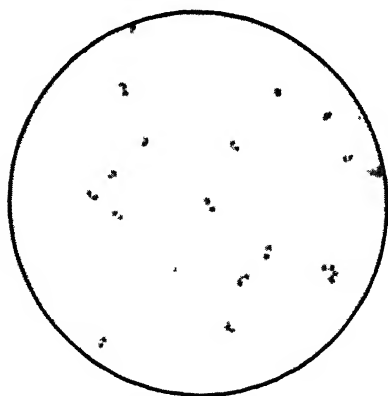


FIG. 59.—PAST. PESTIS FROM YOUNG AGAR CULTURE ( $\times 950$ ).

and somewhat irregular edges. A culture on an agar slope is translucent and the growth, when touched with a platinum wire, is sticky. In older cultures a certain irregularity in the size of the colonies, almost suggestive of impurity in culture, is rather characteristic. Gelatin is not liquefied by the growth of *Past. pestis*.

When grown in broth the organisms fall to the bottom of the tube as a granular deposit, leaving the upper part clear. If, however, a little oil is floated on the broth, the growth starts from the lower surface of the oil drops and long delicate filaments, composed of masses of bacilli, grow downwards towards the bottom. To secure this stalactite type of growth it is essential that the flask should remain absolutely steady, as the slightest movement is sufficient to detach the threads. In broth the bacilli occur most commonly in long chains. No indol is produced by the growth of *Past. pestis* in broth or peptone water. The organism produces acid without gas in media containing glucose, maltose and mannitol. No fermentation of lactose, saccharose or dulcitol is found.

The resistance of the organism against heat, drying, and antiseptics is not especially marked. It withstands, however, a considerable degree of cold. It may survive for one or two weeks in dried sputum and for one month in the cadaver.

Plague is primarily an infection of rodents, particularly of

the rat. In that animal it generally takes the form of an acute and rapidly fatal septicæmia. The lymphatic glands, which are enlarged and congested, are surrounded by hæmorrhagic and oedematous areas. The spleen is enlarged and the liver is mottled owing to the occurrence of small areas of necrosis alternating with hæmorrhagic points. There is generally an excess of fluid in the pleural cavities. More rarely plague may take a more chronic course in the rat. A healthy rat becomes infected most usually by the bite of a flea which has previously bitten a rat suffering from plague septicæmia. Infection also occurs by the devouring of the carcass of a rat dead of the disease.

Human plague is a most serious disease, and from time to time there have been widespread epidemics causing the deaths of millions of victims. It may occur either in mild form, *Pestis Minor*, in which a moderate pyrexia with slight enlargement and tenderness of one or more groups of lymphatic glands are the chief characteristics, or as *Pestis Major*, which may assume one of three types: Bubonic, Pneumonic, or Septicæmic. Bubonic plague, which is of commonest occurrence, commences with a progressive swelling of a group of lymphatic glands, most frequently the inguinal, accompanied by great pain. Marked pyrexia and great prostration are two of the outstanding characteristics of the disease. The pathological condition of the swollen glands is one of intense inflammation with hæmorrhages, leading eventually to necrosis. Not only the glands themselves but also the surrounding tissues are involved, and the bubo, characteristic of the disease, is composed of the enlarged glands of the group fused together with oedematous and hæmorrhagic connective tissue. In the early stages the glands are packed with enormous numbers of coccoid, polar-staining bacilli, and a film prepared from the gland pulp may resemble a pure culture of the organism. When necrosis becomes marked, the number of bacilli decreases and involution forms may be seen. If death does not supervene at this stage, an examination of the glands at a slightly later date may fail to show the presence of any bacilli. In addition to

the primary bubo, one or more secondary buboes may appear in other groups of glands. These are usually much less marked, but they show changes similar to those of the primary bubo. The spleen is enlarged and may show areas of hæmorrhage and

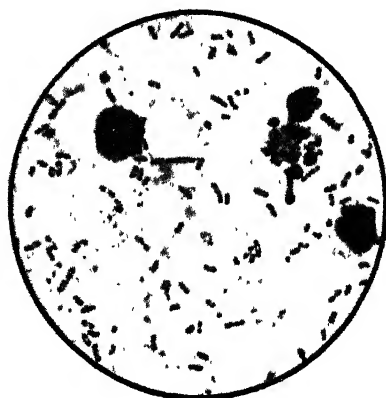


FIG. 60.—*PAST. PESTIS* IN FLUID FROM BUBO ( $\times 800$ ).

necrosis, as may also the liver and lungs. In the spleen, as in the glands, large numbers of bacilli are present. The cells of the liver and kidney show a marked degree of cloudy swelling. An intense septicæmia is commonly present shortly before death, and the number of bacilli in the blood may be so large that they can be found by direct microscopical examination. The bacilli are

frequently present in the blood, even at an early stage of the disease, but in much smaller numbers, and this does not portend, of necessity, a fatal issue. The mortality of bubonic plague varies in different epidemics from 20 to 90 per cent.

In pneumonic plague, the condition is primarily one of rapidly spreading broncho-pneumonia of a hæmorrhagic type. Enormous numbers of the bacilli are to be found in the bloody sputum. This form of the disease is almost invariably fatal and, since the bacilli are freely coughed up, is exceedingly infectious.

In the septicæmic variety, the blood stream is early invaded, and there is but slight localization either in the glands or lungs. In this also death is the usual outcome.

Laboratory animals, particularly guinea-pigs, rats, and mice, are susceptible to infection. As a result of subcutaneous injection in the guinea-pig, there is a local lesion with marked œdema, congestion, and small hæmorrhages. The glands draining the area involved are enlarged and their condition is

very similar to that found in human plague. Congestion and hæmorrhages are found in the internal organs, particularly in the spleen, which is often studded with small pale areas of necrosis resembling, in naked eye appearance, miliary tubercles. The animal generally dies within a week of receiving the injection. The guinea-pig may also be infected by smearing the conjunctiva, or the nasal mucous membrane, or by rubbing the freshly shaved skin of the abdomen with material containing the bacilli. This is a very useful method of isolating plague bacilli from a contaminated source, such as the carcass of a rat suspected of having died from plague. Rats and mice may also be infected by eating food containing the bacilli.

Infection of man in the bubonic and septicæmic types occurs through the skin, either owing to the bacilli contained in dust finding entry through minute abrasions or, in the majority of cases, from the bite of a rat flea which has previously fed on a plague rat. The bacilli in the blood of the rat multiply in the stomach of the flea and are regurgitated at the next feed. The bacilli are also present in the flea's fæces, and by scratching the site of the bite on which the insect has defæcated, the bacilli may be forced into the wound and so enter the body. A flea may remain infective as long as three weeks after partaking of blood from a plague rat. In India, the common host of *Past. pestis* is the rat. The disease is spread from rat to rat by the rat flea. As a result of an epizootic of plague among rats, the number of these is reduced and their fleas transfer themselves to human beings, so causing a human epidemic. Contact and accidental contamination are quite unimportant methods of spread of bubonic plague, the flea is the great intermediary between the rat and man and preventive measures are to be directed chiefly to the elimination and exclusion of rats.

In pneumonic plague, infection is usually direct from patient to victim by means of droplets of sputum containing bacilli. Sporadic cases of pneumonia occur in epidemics of bubonic plague, and these are probably the origin of epidemics of the pneumonic form of the disease. It seems possible that



in a short time, the organisms acquire a preference for the tissue of the lung.

The toxins of *Past. pestis* are almost entirely endotoxins, filtrates of broth cultures being only very slightly toxic. The injection of cultures of the bacillus, killed by heating to 65° C. for one hour, may cause areas of necrosis and hæmorrhages in the internal organs.

Since one attack of plague protects for life, attempts have been made to secure immunity in man by vaccines. The incidence of the disease has been lower in the vaccinated, and in those who did acquire it the mortality has been less, but the protection lasts for only a few months.

Therapeutic sera have been produced in horses in a variety of ways, but the clinical results with most have been doubtful. By long continued immunization of cattle with highly virulent, living cultures a really potent serum seems to have been produced.

The bacteriological diagnosis of the disease in man presents no special difficulties. In places where bubonic plague is endemic, a microscopical examination of fluid, aspirated from a bubo with a hypodermic syringe and needle, is usually satisfactory, as the finding of large numbers of short, fat, polar staining bacilli is sufficient to confirm the diagnosis. It should be noted, however, that in cases which recover, no bacilli may be found microscopically or on culture in the gland fluid in the later stages, although the bubo is still present. In the first case occurring in a non-plague locality, and in the examination of the sputum from a case of suspected pneumonic plague, it is essential to isolate the bacilli and to examine with care their properties. In the identification of *Past. pestis*, the outstanding points are characters of growth on agar, broth, and salt agar, microscopical appearance, agglutination with specific serum, and pathogenicity for animals. During the disease the patient's serum acquires the property of agglutinating the bacilli in a dilution of from  $\frac{1}{10}$  up to  $\frac{1}{100}$ . This may be of some service in establishing the diagnosis in recovered cases.

The examination of carcasses of rats is an important point

in the sanitary control of the disease. An experienced observer can frequently decide, by naked eye examination, if the animal has died from plague. For a complete proof, the isolation and identification of the bacilli are essential. Where putrefaction has occurred ordinary cultural methods usually fail, but *Past. pestis* can be isolated by making use of the fact that the organism can penetrate the nasal mucous membrane or the shaved skin of the guinea-pig, the majority of other bacteria failing to do so.

Occasionally the carcass of a rat may show lesions somewhat suggestive of plague from which a very similar organism, *Past. pseudotuberculosis*, may be isolated. The distinguishing characteristics of this organism are that it may ferment saccharose, a carbohydrate never fermented by *Past. pestis*; that it is not virulent for white rats, for which *Past. pestis* is pathogenic; and that in cultures grown at 18° to 26° C. it is motile (but not if grown at 37° C.), while *Past. pestis* is non-motile.

### Other Members of the Genus *Pasteurella*.

Other species of the genus *Pasteurella* (hæmorrhagic septicæmia group) are pathogenic for a variety of animals, producing septicæmia, but, for the most part, do not infect man. Among these are *Past. avisepctica* (birds), *Past. suisepctica* (pigs), *Past. bovisepctica* (oxen) and *Past. levisepctica* (rabbits).

*Pasteurella tularensis* is another member of this genus. It is smaller than *Past. pestis* and possesses a capsule. Primary cultures are rather difficult to obtain, medium containing blood, glucose, and 0·1 per cent. of cystine being most successful. The colonies are small and viscid. The organism is responsible for a disease occurring naturally in ground squirrels, rabbits, hares and other rodents and possibly also in sheep, cattle, grouse, and quail. Mice, guinea-pigs, and rabbits can be infected experimentally. The disease in human beings, which is known as tularæmia, was first observed in America, but, later, cases were recorded in Japan, Siberia,

Russia, Scandanavia, and Central Europe. Several bacteriologists elsewhere have been infected from laboratory cultures. Infection may take place by inoculation, through the unbroken skin, through the conjunctiva, and possibly by inhalation in those handling animals suffering from the disease, and also by the intermediary of blood-sucking insects—the deer fly (*Chrysops discalis*), the wood tick (*Dermacentor andersoni*), and others which are responsible for its spread among animals.

In animals the disease closely resembles plague and is very fatal, but in man it is milder and rarely fatal, although of long duration. It may resemble typhoid fever or present, as its most striking features, a primary ulcer at the site of inoculation and glandular enlargements which may progress to suppuration. The serum of a human case agglutinates the bacillus strongly, occasionally up to 1/800, and may show some cross agglutination with *Br. abortus*, which suggests that possibly the organism should be considered a member of the genus *Brucella*. Direct cultures from human cases have usually been unsuccessful. The best method of isolating the organism is to infect a guinea-pig with material from the human lesion and obtain a culture from that animal.

## CHAPTER XXXV

### HÆMOPHILIC BACTERIA

#### **Hæmophilus influenzae.** (*B. influenzae* (Pfeiffer).)

Pfeiffer, 1892.

THE influenza bacillus of Pfeiffer is one of the most minute of the ordinary bacteria, measuring from 1 to  $1.5\mu$  by  $0.2$  to  $0.3\mu$ , but almost coccal forms are common in body fluids and filaments may occur in culture. It is found in very large numbers in the sputum of many patients in the early stages of the disease, but experienced observers have failed to detect its presence after careful search in a considerable proportion of cases of clinical influenza. When present, it is found lying singly, in pairs, or most commonly in clusters but never in chains, either between or within the leucocytes in the sputum. It is non-motile, has no capsule, and does not form spores. It is Gram negative and stains less readily than the majority of bacteria, the most successful stains being dilute carbol fuchsin or alkaline methylene blue, applied for a longer time than usual. Frequently the bacilli show bipolar staining, the central portion taking the stain less intensely than the ends.

The bacillus is strictly aerobic and will grow only at, or near, body temperature. It will not grow on plain agar. On blood agar colonies are just visible as very minute clear drop-like dots at the end of twenty-four hours. On the next day the colonies may have a diameter of 1 mm., but are frequently smaller; more profuse growth is obtained on "chocolate medium," that is blood agar, the tubes of which, after the addition of the blood, are heated to  $100^{\circ}$  C. for a few minutes. On this medium the colonies are much larger, grey in colour, and more opaque.

Two factors, X and V, are necessary for the cultivation of *H. influenzae*. X, in blood agar or chocolate agar, is derived from hæmoglobin, but is not actually that substance. It may be methæmoglobin or hæmatin, and iron-containing substances present in fruit and potato can be used instead. This factor is thermostable, and very minute amounts of it suffice. V is present in fresh animal and vegetable tissues, in blood and yeast, and has been identified with cozymase. It is also present in cultures of most bacteria, and its production by staphylococci is illustrated by the phenomenon of satellitism, so characteristic of *H. influenzae*. This is the great increase in the size of colonies of the organism on blood agar in the vicinity of a staphylococcus colony.

In cultures the bacilli exhibit considerable pleomorphism. On chocolate medium they tend to remain short, though usually definitely bacillary; on blood agar longer and even filamentous forms are seen. In blood broth a slight white growth occurs at the bottom of the tube. Some strains produce indol. Most strains ferment glucose and some maltose and saccharose.

Pfeiffer's bacillus dies easily as the result of drying. In moist sputum and in cultures its vitality does not persist for more than a few days. It is easily killed by heat and antiseptics.

The organism is but slightly pathogenic for animals and in none is it possible, by inoculation of cultures, to produce a disease having any great resemblance to human influenza. By direct injection into the lungs of monkeys an acute respiratory disease with pyrexia and broncho-pneumonia may be produced. Intravenous inoculation of rabbits with living or dead cultures causes dyspnœa, muscular weakness and frequently death. Local inoculation in the upper respiratory tract of dogs with a strain, the virulence of which has been exalted by passage, produces an acute respiratory disease with some broncho-pneumonia.

That Pfeiffer's bacillus was the cause of influenza in man was commonly accepted until the epidemics of 1918 and 1919. During these years many bacteriologists, in many lands,

investigated the bacteriology of the disease. Some, in certain places, found the bacilli in practically every case. Others, however, failed in many or even in the majority of cases to isolate the organism. Experimental inoculations of men with pure cultures did not generally produce the disease; at the most some pharyngitis and bronchitis resulted from the application of cultures to the mucous membrane of the respiratory tract. Although probably not the cause of influenza, Pfeiffer's bacillus is definitely pathogenic for man. It is, for example, a not uncommon cause of meningitis. It is now, almost universally, accepted that influenza is due to a virus but, in many respects, it resembles the influenza of swine. This disease is due to the combined action of a virus which, in its pathogenic action on mice and ferrets, closely resembles the virus believed to be the cause of the human disease and a hæmophilic bacillus, very similar to *H. influenzae*.

***Hæmophilus pertussis.* (*B. pertussis*.)**

Bordet and Gengou, 1906.

*H. pertussis* is an organism very similar to Pfeiffer's bacillus. It is inclined to be slightly larger than this bacillus, which it resembles in staining peculiarities and in characteristic grouping.

It is more difficult to obtain in primary culture than *H. influenzae* and, for culture, it is essential to have sputum from a case in the early stages of whooping cough, as the organisms, at first very numerous, disappear later. The medium recommended by Bordet and Gengou, which is potato-extract glycerol-agar with up to 50 per cent. of blood, is superior to plain blood-agar. The colonies after 72 hours incubation are smaller than those of Pfeiffer's bacillus but otherwise similar. Sub-cultures may, however, grow much more luxuriantly than those of *H. influenzae*, the colonies becoming sticky, more opaque, and grey or slightly brown in colour. Hæmoglobin is of assistance in securing growth of the *H. pertussis*, but it is not absolutely essential and the organism can grow in the

absence of both X and V factors. Old cultures may even grow on plain agar.

In the early stages of whooping cough the bacilli may be found in enormous numbers in the sputum, particularly in the thick viscid secretion obtained from the smaller bronchioles at the end of a paroxysm of coughing. They are also seen in sections of the lung lying on or between the cells of the mucous membrane of the bronchioles and alveoli. When correct technique is adopted the organisms are found in almost every case of the disease. Plates of Bordet and Gengou's medium on which the patient coughs are now frequently used for the diagnosis of the disease. *H. pertussis* is distinctly toxic for some animals, the intravenous injection of autolysed cultures killing rabbits in from twenty-four to forty-eight hours. Only endotoxins are produced. Intra-tracheal inoculation of dogs and monkeys with the living organism produces a catarrhal inflammation of the respiratory mucous membrane, with pyrexia and occasionally patches of broncho-pneumonia, death occurring in a number of cases in from two to three weeks. The appearance of the bacilli in the bronchi of the animals is similar to that in the human disease.

While the bacillus has not been definitely proved to be the causal organism of whooping cough, the evidence is largely in favour of this. The fact that the bacillus has been found in the sputum of a small number of adults suffering from chronic bronchitis is no weighty objection.

During an epidemic only one serological type of bacillus is found. The serum of a convalescent agglutinates the organism and complement-fixing substances are present in it. The work of Gardner and Leslie has cleared up some of the difficulties in connection with the serology of *H. pertussis*. In artificial culture, on unsuitable medium, the bacillus undergoes antigenic changes, passing from Phase I (that present in the sputum) to Phase II, which, like Phase I, is smooth and toxic. Later it becomes rough and non-toxic, passing through Phase III to Phase IV. In the later, rough phases the bacilli are not agglutinated by convalescent serum.

Vaccines have been used both therapeutically and prophylactically and, in the hands of some, appear to have given good results. For their preparation the bacilli in Phase I must be used.

Two organisms, somewhat similar to Pfeiffer's bacillus, the Koch-Weeks bacillus and the Morax-Axenfeld bacillus, are associated with acute and subacute angular conjunctivitis respectively and are believed by some to be causative of these conditions.

### **Hæmophilus ducreyi.**

(*Bacillus of Soft Chancre, Ducrey's bacillus.*)

Ducrey, 1899.

This bacillus measures from 1·5 to 2 $\mu$  by 0·5 $\mu$ . In a soft chancre, and in the pus of a bubo, it commonly occurs in chains of from three to twenty bacilli, but solitary bacilli are seen either free or within pus cells. It is Gram negative, and frequently exhibits polar staining.

The bacillus is difficult to cultivate at first, and the most favourable medium is either a rich blood agar, or rabbit blood which has been allowed to clot and to express its serum and has then been heated to 55° C. for five minutes. In later cultures, neither X nor V factor is necessary for its growth.

Cultures on inoculation produce in man and in certain monkeys a typical soft chancre.

The organism is a delicate one, easily killed by exposure, and infection usually spreads directly from individual to individual, particularly in coitus.

Soft chancre must be distinguished from lymphogranuloma inguinale (climatic bubo), due to a virus, and from granuloma venereum, due to a leishmania.



## CHAPTER XXXVI

### BACTERIA CAUSING UNDULANT FEVER

**Brucella melitensis.** (*B. melitensis*.)

Bruce, 1887.

UNDULANT fever (formerly known as Malta fever) is caused by a very minute bacillus, the *Br. melitensis*. The organism, which in preparations made from the body is ovoid, measures about 0.2 by 0.4 $\mu$ , and was formerly described as a coccus (*Micrococcus melitensis*), but it is definitely bacillary in form. It occurs most commonly either singly or in pairs, rarely in short chains. It is non-motile and does not form spores. It stains well with any of the ordinary stains but is Gram negative.

*Br. melitensis* is aerobic and facultatively anaerobic. It grows slowly in artificial culture, and forty-eight hours or more may elapse before the colonies are visible, while the maximum size (about 3 mms. in diameter) is not attained until after about one week's incubation. The colonies are round, even, and somewhat elevated. In colour they are whitish; but when examined by transmitted light they are fairly transparent, with rather opaque centres of a yellowish or brown colour. It grows best at 37° C., but slow growth also occurs at 20° C. Gelatin is not liquefied and no sugar is fermented by its growth. In broth a general turbidity is slowly produced but, after some time, a flocculent deposit is found at the bottom of the tube. The organism resists cold and drying very well and survives in dry dust for some months. It has no remarkable resistance against heat or chemical antiseptics.

In man the disease, which is characterized by headache, sweating, pains in the joints, orchitis, constipation, enlarged spleen and irregular pyrexia, may persist for weeks or even months. The mortality is small, not exceeding about 2 per

cent. In fatal cases the most marked feature is enlargement of the spleen, which is congested and soft. The organism can, almost invariably, be found by blood culture in the early stages of the disease. It is present in large numbers in the spleen, from which it may be isolated by aspirating a small amount of bloody fluid with a syringe and fine needle. It is also present in lymphatic glands, the liver, gall-bladder and kidneys. In some cases the organism has been found in the urine, and occasionally in the faeces.

The disease may possibly be contracted by close contact with a patient; it certainly may through skin wounds and abrasions, as happened in a number of laboratory infections. The commonest method, however, is by drinking the milk of infected goats. A high percentage of the goats in Malta suffer from the disease, which at first may produce abortion but later has little effect, the organism remaining in the mammary gland and being excreted in large numbers in the milk and also in the urine. It is to the drinking of such milk that the majority of human cases are to be ascribed. The obvious preventive measure is to boil or pasteurize all suspected milk before using it for food.

Other animals—cows, horses and sheep—are susceptible to the disease, but to a less extent. Rabbits, guinea-pigs and mice are relatively immune, but the hamster is susceptible. In the monkey a disease exactly comparable to that of man may be produced, either by feeding or by rubbing cultures on the scarified skin.

A diagnosis may be made by culture, either from the blood or spleen or, less commonly, from the urine. All cultures, especially those from the blood, should be incubated for several weeks as, in many cases, the organism develops slowly. The fact that agglutinins are present in the blood of the patient within a week of contracting the disease renders the agglutination test a useful and reliable method of diagnosis. Agglutination of *Br. melitensis* by the serum of a patient, diluted  $\frac{1}{100}$ , is almost diagnostic, but much higher titres, up to  $\frac{1}{500}$  or more, may be met with. Diagnosis of the disease in animals may be made in the same way.

Vaccines have been used prophylactically, and seem to afford a definite protection to those exposed to infection.

***Brucella abortus.* (*B. abortus.*)**

Bang, 1897.

Two types of *Br. abortus* are known—the bovine and the porcine. The latter is sometimes called *Br. suis*. These two are so similar morphologically, culturally, and serologically both to one another and to *Br. melitensis* that they should be regarded as variants of one species, *Br. abortus*, modified by passage through the cow, the pig, and the goat.

The bovine type is of most common occurrence throughout the world, the porcine and the caprine types being more restricted in their distribution. The naturally occurring animal diseases are similar, but abortion results from infection most commonly in the cow.

When freshly isolated from animal or man the three types show certain cultural differences, of which the most important is that the bovine type can only be cultivated in air to which 10 per cent. of carbon dioxide has been added. Other differential points are the resistance of *Br. melitensis* to the presence in the medium of thionin, pyronin, methyl violet and fuchsin; the resistance of the porcine type to thionin with sensitivity to the other dyes; and the resistance of the bovine type to pyronin, methyl violet and fuchsin with sensitivity to thionin. For guinea-pigs, *Br. melitensis* is least pathogenic, the bovine type slightly more, and the porcine most pathogenic.

Antigenically the organisms, when freshly isolated (and in the smooth phase) present slight differences. They can be distinguished serologically but only by the antibody absorption technique. Old cultures become rough and agglutinable by heat. In this condition (formerly called para-melitensis and para-*abortus* strains) their original type cannot be determined.

Both the porcine and bovine types of *Br. abortus* can cause undulant fever in man. This is, typically, a long-continued pyrexia, often with remissions, which may be

accompanied by joint pains, skin rashes, and other symptoms. There is little doubt that the porcine type is more pathogenic for man than the bovine, but difficulty may be caused owing to cows being infected with the porcine type. Human infection may result from attending on infected animals after abortion or at other times, or from handling the flesh of such animals. It may also result from drinking milk from infected cows. Since about 10 per cent. of cows in the British Islands excrete the organism in their milk, it is difficult to understand why so few human cases occur there as compared with other countries—America, Denmark, and Rhodesia—where the disease is common.

The only really satisfactory method of diagnosis in man is by isolation of the organism from the blood. Cultures of blood should be incubated in air containing 10 per cent. of  $\text{CO}_2$  for at least ten days. Failing this, a diagnosis is often made by finding that the patient's serum agglutinates *Br. abortus* in a dilution of  $\frac{1}{100}$ . Since it is not rare to find similar or higher titres in healthy men, who give no history of fever, we do not consider such a conclusion valid. If blood cultures are negative, we do not think a diagnosis of undulant fever of the *abortus* variety should be made unless symptoms are typical and the serum agglutinin titre is at least  $\frac{1}{500}$ .

## CHAPTER XXXVII

### THE TUBERCLE BACILLUS

***Mycobacterium tuberculosis* (hominis).**

Koch, 1884.

***Mycobacterium tuberculosis* (bovis).**

Th. Smith, 1896.

***Mycobacterium avium*.**

Strauss and Gamaléia, 1891.

THERE are three chief types of Tubercle Bacilli—the Human, the Bovine and the Avian. They present sufficient individual peculiarities to be considered as separate types and not merely as modifications of the same organism. Up to the present, none of the many experiments undertaken has succeeded in transforming an organism from one type into another. We will first consider the morphological and cultural characteristics of the three types separately, and will then deal with tuberculosis in general and the pathogenic properties of the three.

The human type of tubercle bacillus is a thin, straight or slightly curved bacillus measuring from 2 to  $5\mu$  by  $0.3\mu$ . The bacilli are found in material taken from the body singly, in pairs usually arranged at an angle, or in clusters in which the individuals are commonly more or less parallel in arrangement. They may stain uniformly or may present a beaded appearance, due to the alternation of darkly staining portions with those which are either unstained or but faintly stained. In old cultures clubbed or filamentous forms may be present, and these latter may show branching. The bacilli are non-motile, possess no capsules and do not form spores. The usual stains cannot be relied upon to stain tubercle bacilli, unless the time is increased or heat is applied. When, however, the bacilli are stained, they very strongly resist decolorization

with alcohol or with mineral acids. These peculiarities—the difficulty in staining and the resistance against decolorizing agents (the so-called acid-fast and alcohol-fast properties)—are most important points in distinguishing them from the majority of other bacteria: the reason is probably the considerable amount of waxy and lipoidal substances present in their bodies. The most useful staining method is the Ziehl-Neelsen method, in which warmed carbol-fuchsin is the stain and the decolorizer either 20 per cent. sulphuric acid solution in water or 3 per cent. hydrochloric acid in spirit. (*See Plate I.*) When the bacilli are stained by methyl violet they are found to be Gram positive. Under certain conditions young bacilli appear to be not acid-fast, and in tuberculous pus it may be impossible to detect acid-fast bacilli, and yet the material may cause tuberculosis in animals when injected. Some workers believe that there is a filterable type of the tubercle bacillus.

To obtain cultures of the tubercle bacillus special media and prolonged incubation are necessary. The slow growth prohibits the plating method commonly employed for the isolation of other bacteria, because the medium would become dry or would be overgrown by other organisms before the tubercle bacilli could develop. Cultures may be obtained by spreading material over the surface of special medium in a tube, provided no other organisms are present. To prevent the drying of the medium in the tube, it should be sealed by pouring melted paraffin wax over the plug. The methods used for obtaining cultures from sputum will be described later.

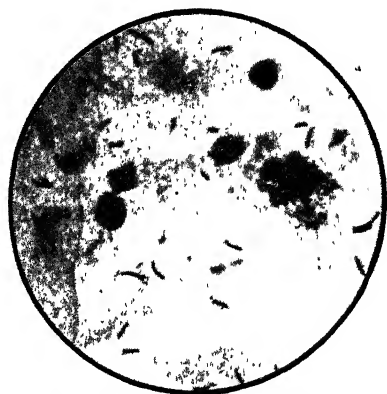


FIG. 61.—TUBERCLE BACILLI IN SPUTUM ( $\times 800$ ).

Coagulated serum or egg media are the most useful for obtaining primary cultures. Egg medium may consist of eggs and water alone, the mixture being coagulated by heat, or may be modified by the addition of broth, glycerol, or other substances. For later cultures glycerol-agar, glycerol-

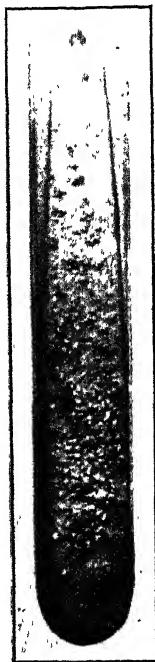


FIG. 62.—CULTURE OF TUBERCLE BACILLUS (HUMAN TYPE) ON PETROFF'S MEDIUM ( $\times \frac{1}{2}$ ).

broth, or glycerol-potato may be used. While the addition of glycerol greatly promotes the growth of subcultures of the human type of tubercle bacillus this is not invariably true of primary cultures which, even in the case of the human type, may be inhibited by the presence of glycerol in the medium. For isolation, therefore, it is advisable to use both plain and glycerol egg media. Tubercle bacilli grow best aerobically at a temperature of  $37^{\circ}\text{C}$ . : under anaerobic conditions the growth is always slight. Colonies of the human type bacilli begin to appear in from ten to fourteen days as minute greyish points. In subculture in from three to four weeks an extensive growth is generally obtained, which spreads over the greater portion of the surface of the medium as a dull, dry, wrinkled, or warty film. If plain egg medium is used, the colour is grey, but, if glycerol be present, the colour may be cream, brown, or pink.

When touched with a wire it is found to have considerable cohesiveness, and an isolated fragment is difficult to break up in order to prepare a film. On glycerol broth, when growth is started by floating a thin piece of

the culture film on the surface, a pellicle forms which spreads over the surface of the broth and for a little way above the surface on the sides of the flask. The pellicle increases in thickness and becomes wrinkled, closely resembling the growth on a solid medium.

In all essentials the bovine type resembles very closely

the human. It is, however, inclined to be rather thicker and shorter and also to show less variations both in size and appearance.

It has been noticed that on suitable culture media the human type grows freely. To this peculiarity the term "eugonic" is applied. The bovine type, on the other hand, is spoken of as "dysgonic" since, in earlier generations at least, the growth is never luxuriant. On egg or serum media in subculture it produces a thin, smooth, translucent film which is but slightly granular and is less dry than the human. This growth, when picked up, is found to be much less cohesive than the human. The growth of the bovine type is not assisted by the presence of glycerol in the medium; indeed glycerol may have a considerable inhibitory effect in earlier generations. Glycerol, however, exercises less influence on cultures of long isolated strains of the bovine type of bacillus.

The avian type of tubercle bacillus, while in morphology and staining characteristics it closely resembles the preceding, shows considerable differences in culture. It grows luxuriantly at  $42^{\circ}$  to  $43^{\circ}$  C., a temperature sufficiently high to prevent any development of either the human or the bovine types. The growth is much more profuse and is definitely moist, in distinction to the dryness of cultures of the human type. In colour it is inclined to be yellow or orange rather than greyish white.

It has been suggested that what we have for long regarded as the typical tubercle bacillus—an acid- and alcohol-fast organism growing in the form of rough colonies—may be merely a variant of low virulence, whereas the virulent bacilli



FIG. 63.—CULTURE OF TUBERCLE BACILLUS (HUMAN TYPE) ON SURFACE OF GLYCEROL-BROTH.



are slightly, if at all, acid-fast and form smooth colonies. It is not yet possible to give a definite opinion on this suggestion.

Tubercle bacilli of whatever type show considerable resistance to unsuitable environments. In the dry state they can survive exposure to 100° C. for twenty minutes or more, but when moist a temperature of 60° C. kills in less than that time. In dried sputum the bacilli may remain viable for weeks or even months. They remain alive when exposed to 0° C. for several months, and still retain their pathogenic properties after an exposure to gastric juice for six hours. Their resistance to chemical agents, such as sodium hydrate or sulphuric acid, is shown by the chief methods used to free

them from other bacteria in order to obtain pure cultures. They are fairly rapidly killed by exposure to direct sunlight.

Tuberculosis in man is due chiefly to the action of the human type of tubercle bacillus, to a lesser extent to the bovine type and practically never to the avian. The fundamental lesion in the bodies of man or animals is the tubercle.

Since the pathology of



FIG. 64.—TUBERCLE BACILLI IN SECTION OF LUNG ( $\times 950$ ).

tuberculosis is fully considered in works on pathology it need not be detailed here. It is sufficient to recall the three types of cell found in the tubercle—the epithelioid, the giant and the lymphocyte. As the tubercle increases in size, necrosis of the central portion occurs, owing partly to the absence of blood supply but chiefly to the poisonous products of the bacilli. This process is spoken of as caseation, and the cheesy substance in the centre of the tubercle is called caseous material.\* Widely different pathological pictures result from the action of tubercle bacilli on different parts of the body, but the primary

abnormality, the tubercle, is much the same everywhere. When the disease becomes retrogressive the tubercles are surrounded by fibrous tissue and so walled off, and at a still later stage the caseous mass may become calcified. In addition to the local result of invasion by tubercle bacilli (the production of tubercles) general effects are produced, especially pyrexia, sweating and wasting, as a result of the spreading by the circulation of some of the poisonous products of the bacilli. These effects are often accentuated by secondary infection with other organisms, which is very common in tuberculosis, particularly of the lung.

Usually tubercle bacilli may be found in tuberculous lesions, but the number is largest in acute and rapidly spreading disease, while in chronic tuberculosis very few bacilli may be found. When the disease is an "open" one, that is where one or more foci are in direct or indirect contact with the exterior of the body, as in lung, bowel or kidney disease, bacilli may leave the body, and it is by such means that the disease is spread. It has been calculated that a man suffering from phthisis may expectorate more than a thousand million tubercle bacilli in twenty-four hours. Man is moderately susceptible to the human type of bacilli, the exact result of infection depending partly on the virulence and number of the invading organisms, but much more on his individual powers of resistance and general health. He is much less susceptible to the action of the bovine type, and in adult life fresh infections with this type are rarely encountered. In the case of young children, however, the condition is different, for these possess but little resistance against large doses of the bovine bacilli. The great majority of cases of lung tuberculosis are due to the human type, but in tuberculosis of glands, of the abdomen and of bones and joints in children the incidence of the bovine type is high in communities supplied with milk from tuberculous cows.

In the case of other animals the bovine type is much the more virulent. The calf is almost completely immune to the human type, only a slight local lesion being produced by its injection. The rabbit, injected intravenously with 0.01 mgm.

of a culture of the bovine type, dies in about a month with widespread progressive tuberculosis. A similar injection of the human type is usually not fatal and only causes slight lesions, chiefly in the lungs and kidneys. Subcutaneous injection of the human bacilli in the rabbit fails to kill and produces a local lesion and small lesions in the lungs and kidneys; the bovine, on the other hand, causes death in 2 to 3 months with generalized tuberculosis. This difference between the two organisms in their pathogenicity for animals, particularly for the rabbit, is one of the characteristics which enable them to be distinguished. The guinea-pig is susceptible to both types, but distinctly more so to the bovine. When injected subcutaneously, death results in about 3 months with the bovine type and rather later with the human. In both cases there is to be found a local caseous lesion, involvement of the various groups of lymphatic glands draining the area, and usually of the spleen, liver, and lungs. Inoculation of a guinea-pig is one of the methods used for isolating tubercle bacilli and also for their identification in suspected material.

The tubercle bacillus is a strictly pathogenic organism and, apart from laboratory cultures, appears to be incapable of acting as a saprophyte. Infection is, therefore, from case to case either directly or indirectly. The bacilli in the case of man are excreted chiefly from the respiratory, intestinal, and urinary tracts, especially the former. The bacilli may be carried directly from a case to a fresh victim in droplets of sputum but probably more commonly in dust composed of dried sputum containing bacilli. The prolonged vitality of the bacilli in the dry condition and the common habit of promiscuous expectoration are the two chief factors which go to make tuberculosis the scourge it is to-day. In the case of the human variety the bacilli passing either from the bladder or intestine are much less commonly the cause of fresh cases. In the case of animals, in which infection occurs as a result of contamination of pastures, the intestinal route is of greater importance than it is in man. The chief agency by which the bovine bacilli are conveyed to man is the milk of infected cows. In these animals tuberculosis of the udder is a fairly common

condition, but even when the udder has no trace of the disease the bacilli from other parts of the body may be excreted in the milk. Under dirty conditions cow's milk contains a considerable amount of fæces and, when tuberculosis of the intestine exists, the milk may contain a large number of the bacilli. The flesh of tuberculous animals can be responsible for only a small number of cases of tuberculosis in man in countries where raw meat is not a common article of diet. Further, it is rare for the flesh (*i.e.* muscle) of animals to become tuberculous, even when the glands are extensively involved.

The route of infection

in man may be either by inhalation, by ingestion, or by the skin. That inhalation is the commonest of these there can be little doubt, but as to the exact method by which the bacilli reach the lung, the usual site of the disease, there is still little agreement. In some cases they are carried in the air to the deeper parts of the



FIG. 65.—TUBERCLE BACILLI IN DEPOSIT FROM URINE ( $\times 950$ ).

respiratory tract. Another method appears to be by penetration of the epithelial lining of the upper part of the tract and thence, by the lymph stream, to the mediastinal glands and the lung itself. The tonsils may be one of the most important portals, and as a result may show evidence of tuberculosis, or more commonly may be unaffected. Bacilli ingested either in dust or in food may cause tuberculosis of the intestine, but there is no doubt that they are capable of penetrating the mucous membrane without inflicting any injury and of causing tuberculosis either of the mesenteric glands or of other parts. Tuberculosis due to ingestion occurs most commonly in children and is generally due to the bovine type of bacillus. The source of the bacilli is very

frequently the uncooked milk of tuberculous cows. Skin infection may occur in butchers, veterinary surgeons, and others who handle infected material. This form of the disease tends to remain localized in the skin and is very chronic. Lupus vulgaris is another type of tuberculous skin lesion which occurs most commonly about the face. The mode of infection in these cases is uncertain.

The widespread distribution of tuberculous lesions throughout the body in advanced cases can only be explained by the common occurrence of the bacilli in the blood stream, but the claims of certain workers to have isolated tubercle bacilli from the blood in a large proportion of cases is not generally accepted.

Tubercle bacilli in the body cause injury owing to the various toxic substances produced by their growth. Although filtrates of broth cultures may produce, on injection, toxic effects such as fever and an acute inflammatory reaction, it is doubtful if the bacilli form any true extracellular toxin. The toxins produced, of which there are probably several distinct varieties, are entirely endotoxins. Dead tubercle bacilli, when injected into the animal's body, are capable of producing tubercles, a point of practical importance when animal inoculation is used for the identification of living tubercle bacilli: unless the dose is large, caseation does not occur. Endotoxins are also apparently responsible for the cachexia so commonly found in tuberculosis.

The problem of immunity in tuberculosis is one of great difficulty and complexity. The disease tends to be of great chronicity and, even in its most acute forms, is much more prolonged than the majority of bacterial diseases. While the disease in very many cases becomes arrested or even cured, we have little evidence to show that cure is due to specific antibacterial or antitoxic substances produced by the body in response to the stimulus of the bacilli. Rather, cure seems to depend on the general health and resistance of the body and to be promoted, to a great extent, by such general factors as nutrition and environment. Certain facts suggest the possibility that, as the result of overcoming infection with the

bovine type of bacillus, there is acquired some protection against later human type infection; but these facts may be capable of a different interpretation. In the human body, during the disease, certain types of antibodies may be found—agglutinins, precipitins, opsonins and complement fixing substances—but their exact significance cannot, as yet, be claimed to be thoroughly known. Complement fixation, for example, may be strong in those showing no clinical trace of tuberculosis and weak or absent in those in a very advanced stage of the disease. In tuberculosis instead of the patient tolerating well considerable amounts of the tuberculous toxins as one would expect, there is developed a very great sensitiveness to them. So we find that a patient with tuberculosis may be rendered very gravely ill, or may even die, as a result of an injection of an amount of "Tuberculin" which would be almost without effect on a healthy individual. This hypersensitiveness is also seen in the various local tuberculin reactions considered later.

Tuberculin was the name given by Koch to a filtered and concentrated glycerol-broth culture of tubercle bacilli. This is often referred to as Old Tuberculin (T.). The active principle of tuberculin, which is protein in nature, may be precipitated and purified. Other preparations of the tubercle bacillus—T.O., a watery extract of bacilli disintegrated in an agate mill; T.R., the residue from the preparation of T.O.; and B.E., a suspension of ground bacilli in 50 per cent. glycerol—are frequently called tuberculins, but the term should be reserved for material produced by Koch's original method. Tuberculin was designed both for diagnosis and treatment. It was, however, brought into disrepute by its careless use, whereby severe and even fatal reactions were caused. Now it is being used more rationally, and in many cases good results are being reported. Tuberculin is not, however, a cure for tuberculosis, although its proper use may assist other therapeutic and, more important, hygienic measures, especially in localized tuberculosis of kidneys, glands, bones, and joints. Care should always be taken to start with a very small dose and to increase the dose very slowly at later injections, avoiding

any but the slightest of reactions. The hypersensitiveness of the tuberculous patient to the bacilli or their products may be shown by reactions which are either local (at the site of injection), focal (at the site of the disease), or general (pyrexia and headache). The focal and general reactions would appear to be due to the action of the injected tuberculin on the tubercles, in which there follows hyperæmia, softening and liberation of poisonous substances into the circulation. The original aim of tuberculin therapeutics was to provoke this focal reaction, for following it there is an active stimulation of the surrounding cells to proliferate and this may lead to encapsulation of the diseased focus. The focal reaction must, however, be kept quite slight, as otherwise there may be caused an acute exacerbation of the disease with rapid spread.

Allergy is the basis of several diagnostic tests. The original one was performed by the subcutaneous injection of tuberculin, but this is too dangerous to use for man. 1.0 c.c. of tuberculin injected into a new-born infant is practically without effect, but a much smaller amount (0.01 to 0.0001 c.c.) in a tuberculous subject may cause very severe local, focal, and general reactions. The test now most commonly used in human beings is that of Mantoux, in which 0.1 c.c. of a  $\frac{1}{10000}$  dilution of tuberculin is injected intradermally. In the positive, redness and oedema develop within a few hours and reach a maximum next day. Vesication may occasionally occur. If there is no reaction the test should be repeated, using 0.1 c.c. of a  $\frac{1}{1000}$  dilution and, if this is negative, 0.1 c.c. of a  $\frac{1}{100}$  dilution. The test suffers from one great drawback—it is too sensitive. The great majority of adults react positively since the test reveals the presence not only of active disease, but also of a healed or quiescent focus, and at some period of their lives almost all adults have suffered from tuberculosis. It is most valuable in children under the age of five years. Cattle are best tested for the presence of tuberculosis by the intradermal inoculation of tuberculin.

A living vaccine, consisting of a strain of tubercle bacillus (*Bacille Calmette-Guerin*, "B.C.G.") deprived of virulence by prolonged subculture on bile medium, has been employed

extensively in France. It was claimed by Calmette that this vaccine, administered orally in three doses on alternate days in the first ten days of life, prevented, to a very large extent, infants living in tuberculous homes from contracting the disease. B.C.G. is now usually administered by subcutaneous inoculation to older children who are exposed to infection and in whom the tuberculin reaction is negative. The claims are not considered entirely justified by very many bacteriologists of other countries nor is the safety of a living tubercle bacillus vaccine universally admitted.

Various sera, prepared from animals of different species by different methods; have been tried therapeutically, but despite the claims made for them, none has yet survived a rigorous test.

Since the acid- and alcohol-fastness of the tubercle bacillus is the characteristic on which we chiefly rely for its identification, a consideration of the other bacteria with similar staining properties is necessary. The most important of the other acid-fast bacilli is the Leprosy bacillus, which will be considered separately. There are a number of other organisms of which Moeller's grass bacillus and the butter bacillus of Rabinowitsch may be taken as types. These bacilli resist decolorization with acids, but are easily distinguished from the tubercle bacillus by their rapid growth, even at air temperature, on ordinary media. They are practically non-pathogenic for animals, although a large injection may cause a slight local lesion. Another acid-fast organism is Johne's bacillus, which is found in enormous numbers in the thickened mucous membrane of the small intestine of cattle suffering from chronic paratuberculous enteritis. Most important, from the point of view of diagnosis of tuberculosis in man, is the *Smegma* bacillus. This organism is a harmless saprophyte which may be found about the external genitals of both sexes. It is rather shorter than the tubercle bacillus and is described as being acid-, but not alcohol-fast; not much reliance can be placed on these characteristics, since different types vary greatly in these respects, and some resist decolorization with alcohol very strongly.



The finding of acid- and alcohol-fast bacilli of typical shape in material coming from the interior of the body—sputum, cerebro-spinal fluid or pus from an unopened cold abscess—is, for all practical purposes, conclusive evidence of tuberculosis. Fluids such as cerebro-spinal fluid, pleural fluid, or urine, should be centrifuged and films made from the deposit; but even by this method prolonged search may be necessary before bacilli are found, and even in definitely tuberculous conditions no bacilli may be found, particularly in pleural effusions. In such cases the nature of the cells present in the fluid, usually, though not invariably, mononuclear leucocytes, and the absence of growth on ordinary media, suggest a tuberculous infection, even where the bacilli cannot be found. Particular care is required in the microscopic examination of urine or fæces, owing to the possibility of smegma bacilli being present. If, however, acid- and alcohol-fast bacilli of typical morphology are found in a catheter specimen of urine in which pus is present and no growth is obtained in 48 hours on culture, they are, almost certainly, tubercle bacilli. It is doubtful if it is ever correct to report tubercle bacilli as present in fæces without animal inoculation.

Where any material is being examined directly for the presence of tubercle bacilli, it should be borne in mind that it is practically impossible either to over-stain or over-decolorize in the Ziehl-Neelsen method.

Since sputum is the material most frequently examined for the presence of tubercle bacilli, the various methods employed will be considered in some detail. Four methods are available: (1) direct microscopic examination; (2) microscopic examination after concentration; (3) culture; (4) animal inoculation.

For direct examination, smears are made from selected particles of necrotic material. Care must be taken to distinguish these from particles of food. The smears are stained by the Ziehl-Neelsen method.

When direct smears fail to reveal tubercle bacilli, concentration is used. Add to some sputum in a test-tube an equal volume of 0.2 per cent. Na OH, shake, heat for 10 minutes

at 100° C., and centrifuge. Films are prepared from the deposit. In staining these, the treatment with carbol fuchsin should be applied for 20 minutes, as the alkali tends to cause weak staining.

When microscopic examination fails, either culture or animal inoculation or, preferably, both may be attempted.

Culture, if properly done, can be just as reliable as animal inoculation and may give a positive result earlier. Sputum cannot be sown directly on media because the contaminants present in it grow rapidly and usually destroy the slopes. These must, therefore, be killed before cultures are made and, at the same time, tubercle bacilli must be set free from the necrotic material. Both these are accomplished by adding to some sputum in a sterile test-tube an equal volume of 5 per cent. KOH and incubating at 37° C. for 45 minutes. The mucilaginous suspension obtained is sown by means of a Pasteur pipette on at least two tubes of Griffith's plain egg medium and one tube of 5 per cent. glycerol egg medium. The more tubes inoculated, the better is the chance of isolating tubercle bacilli. Drying of the medium is prevented by treating the plugs with melted paraffin wax. The tubes should be examined on the third day for contamination. If the latter is heavy, the original sputum or a fresh specimen should be treated with an equal volume of 3.4 per cent. (by volume)  $\text{H}_2\text{SO}_4$  in distilled water at 37° C. for half an hour. The mixture is then diluted with 10 volumes of sterile saline and centrifuged and the deposit sown on tubes of medium as before. Alternatively, the  $\text{H}_2\text{SO}_4$  treatment may be used at the outset instead of KOH. Tubes should be examined at least once a week with a hand lens ( $\times 6$ ). The colonies, which may be only pin-point in size, appear in from two to four weeks. Smears should be made from suspected colonies, and these should be spread over the slope as soon as they are seen.

Sputum intended for animal inoculation should be treated as for culture since, if injected without treatment, other bacteria present may cause acute infection and death of the animal before tuberculosis has developed. Either method (KOH or  $\text{H}_2\text{SO}_4$ ) may be used, and the mixture, after treat-

ment, must be neutralized (with  $\text{H}_2\text{SO}_4$  or KOH) to litmus or phenol red before injection. A total volume of 4 c.c. may be injected intramuscularly into the thigh of a guinea-pig's hind leg. Guinea-pigs used for this purpose should weigh 300 gms. or more.

In positive cases, the inguinal lymphatic glands become palpable in about three weeks. The animal should be killed at the end of six weeks.

The post-mortem findings in a positive case are as follows : a local caseous or purulent lesion at the site of inoculation ; enlarged superficial and deep inguinal glands on the affected side which may be as large as a cherry and may be solid or caseous ; the superficial inguinal glands on the opposite side may be enlarged ; the lymphatic glands at the bifurcation of the abdominal aorta, the preaortic glands at the level of the kidneys and the portal gland, above and behind the pylorus, may be enlarged and even caseous ; the spleen, which normally measures  $2 \times 1$  cms., is enlarged, sometimes to as much as  $8 \times 5$  cms., dark red in colour and shows a variable number of irregular, white or cream necrotic areas varying in size from 0.5 mm. to 2 cms. in diameter ; the liver, which is enlarged and pale, usually contains only a few irregular cream or yellow areas of similar size ; the lungs may be studded with pinhead or larger irregular foci, cream in colour with green margins.

That the various lesions are tuberculous should be confirmed by smears stained by the Ziehl-Neelsen method. Errors may arise from the existence in the animal of pseudotuberculosis, a disease caused by a Gram-negative, bipolar bacillus, *Pasteurella pseudotuberculosis*.

The above methods of examination may be used for any other material, such as pus, but unless there is gross contamination, treatment with 5 per cent. KOH need only be for 30 minutes at  $37^\circ \text{C}$ .

Saprophytic acid-fast bacilli are frequently found in corks and rubber bungs so that, if either of these are used for sputum containers, they should be thoroughly washed and sterilized before use. Saprophytic acid-fast bacilli do not cause lesions in a guinea-pig resembling those of tuberculosis and do not kill.

While the finding of tubercle bacilli is the only certain method of making a diagnosis it may sometimes fail even with the greatest care. Then the patient's hypersensitiveness to tuberculin may be tested, but it must be borne in mind that a positive reaction may be due to a healed focus and that a negative result may be obtained in very advanced cases. The other indirect tests, agglutination, precipitin, and the opsonic index, cannot be considered reliable owing to the difficulty we find in correlating the laboratory result with the clinical, or even post-mortem, findings. In certain cases a positive result may be present in the apparently healthy and a negative in the tuberculous. Complement fixation tests suffer from the same disadvantages, but in them the obtaining of a satisfactory antigen appears to be of greatest importance. Some observers claim to have obtained very reliable results by this method.

## CHAPTER XXXVIII

### ***Mycobacterium lepræ.* (*B. lepræ.*)**

Hansen, 1879.

Myco. lepræ is an organism which, in many respects, resembles the tubercle bacillus. It is of about the same size, but is inclined to be slightly more slender and is less frequently curved. Its ends are commonly pointed, but occasionally slight clubbing is observed. It may stain uniformly, but beaded forms are of frequent occurrence. It stains with fair ease by any of the ordinary aniline dyes and is Gram positive. When stained like the tubercle bacillus, it resists strongly the decolorizing effect of mineral acids, and so the Ziehl-Neelsen method is commonly used for its demonstration. Generally, although not invariably, it is more easily decolorized with alcohol than the tubercle bacillus. Leprosy bacilli are present in all forms of the disease, but are especially numerous in the nodular form in which certain cells, the lepra cells, are packed with the bacilli which lie in bundles, irregularly arranged. They are less common in the nerve lesions of the anæsthetic form of the disease. They have often been demonstrated in the blood and in the internal organs, particularly the spleen, liver and kidney, and are almost universally present in the nasal mucous membrane and nasal secretions of the diseased.

The present position with regard to the cultivation of the organism is most unsatisfactory. A large variety of cultural methods have been used and a considerable number of distinct organisms have been cultivated from leprosy lesions. Each has been claimed as being the leprosy bacillus, and while the evidence brought forward in support of the claims of some of these must be treated with respect, none has been definitely proved to be the causal organism of leprosy.

Leprosy appears to be a disease exclusively of the human race. In man two distinct types are observed—the nodular or tubercular and the anæsthetic. In the former there is an enormous development of granulation tissue, which occurs either in the form of nodules or as a diffuse infiltration of the skin or mucous membrane. This form is most frequently observed about the face, but the hands and arms or legs are also common sites. Secondly the internal organs, spleen, liver and kidney may be affected. The granulation tissue contains large numbers of mononuclear leucocytes, but its most remarkable feature is the occurrence of “lepra cells,” large cells with clear nuclei. Bacilli may be seen within these cells and also lying free. Caseation is never seen in leprosy nodules, but ulceration is common. In the anæsthetic form, the lesions are situated chiefly in the nerves. The development of granulation tissue is less marked and the effects are mainly due to cicatrization. Anæsthesia and paralysis are the striking features of this form of the disease, and later trophic disturbances help to produce great disfigurement.

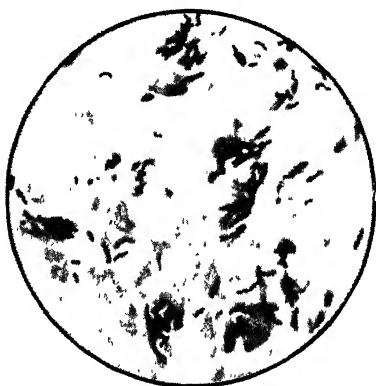


FIG. 66.—MYCO. LEPRÆ IN FILM  
FROM NOSE ( $\times 950$ ).

Leprosy is an exceedingly chronic disease which of itself is not very fatal, death being frequently attributed to tuberculosis or to some other coincident affection. In no other condition is so much disfigurement and crippling produced with so little evident impairment of the general health. In other words, the leprosy bacillus is capable of producing very marked local effects but is only slightly toxic.

The results of animal experiments have been disappointing, since the majority of animals appear to be very resistant

against the *Myco. lepræ*. Successful inoculations, with the production of nodules containing acid-fast bacilli, have been recorded in certain monkeys, rabbits, Japanese dancing mice, and hamsters, but the lesions have never been progressive. Such experiments are rather inconclusive as autoclaved leprosy tissue may produce similar lesions. The finding of acid-fast bacilli locally in animals inoculated with leprosy material must be received with caution, since so many lepers are also tuberculous. The small percentage of successful animal infections, together with the insignificant lesions produced when material containing true leprosy bacilli has been used, renders difficult the identification of organisms found in cultures. Nevertheless similar lesions containing acid-fast bacilli have been produced by the injection of cultures of some of these organisms. It must be realized, however, that many of the non-pathogenic, acid-fast bacilli, when injected in large numbers, may give rise to nodule production, the bacilli occurring in the nodule.

As regards the spread of the disease, the most important fact is that its infectivity is very slight. Both intimate and prolonged contact are necessary for infection. The exact route is unknown, but the fact that the nasal secretions commonly contain large numbers of bacilli is suggestive. One successful inoculation in man has been recorded—a criminal, into whose tissues a portion of a leprosy nodule was planted became a leper; but the reliability of the experiment is open to question, since he may have become infected naturally.

The best results obtained in the treatment of leprosy have been those of Rogers, who uses sodium gynocardate and other derivatives of chaulmoogra oil and hydnocarpus oil by the subcutaneous and intravenous routes.

The finding of enormous numbers of acid-fast bacilli in a human lesion and the absence of effect when a guinea-pig is inoculated with such material is usually sufficient to establish the diagnosis. It should be noted that many of the ulcers may be trophic and may contain no bacilli.

## CHAPTER XXXIX

**Pfeifferella mallei.** *Actinobacillus mallei.*

Löffler, 1886.

*PFEIFFERELLA mallei* is a straight or very slightly curved, round-ended bacillus, measuring from 2 to 5 $\mu$  by from 0.5 to 0.7 $\mu$ . In cultures both long filaments (which may be branched) and short, almost coccal, forms are to be seen. The bacillus is non-motile, has no capsule, and does not form spores. It stains feebly with watery solutions of the basic aniline dyes, but much more deeply if the stain is alkaline (Löffler's methylene blue) or contains a mordant (carbol-fuchsin). One of its chief characteristics is the irregularity with which different parts of the bacillus take the stain. It is Gram negative and not acid-fast.

*Pf. mallei* is aerobic and, under anaerobic conditions, growth is very poor. The optimum temperature of cultivation is that of the body. It grows moderately well on ordinary media, but growth is facilitated by the addition of glycerol to the medium; growth is never very rapid and may not be apparent for several days. Growth occurs more rapidly and profusely on coagulated serum. The growth on potato is rather characteristic: a slimy, yellow, transparent layer appears which resembles a smear of honey; later the colour darkens until in about a week it is reddish-brown or chocolate. *Pf. mallei* ferments glucose slowly: no other carbohydrate is affected.

The resistance of the organism to heat and antiseptics is not striking. It can, however, survive for one or two months in water, an important practical point in connection with horses' drinking-troughs.

Glanders is a disease essentially of the horse family to



which many other animals, including man, are more or less susceptible. The chief feature of the disease is the production, in tissues infected with the bacillus, of the characteristic nodules which are composed of leucocytes (chiefly polymorphonuclear), epithelioid cells and connective tissue. They do not usually produce pus, but generally the central cells degenerate and disintegrate and the nodules become soft and break down, forming ulcers. In the more chronic nodules the connective tissue development is exaggerated and the leucocytic invasion is less marked. Bacilli are fairly plentiful in the acute lesions, mostly extracellular, but a few lie within the leucocytes. It may be impossible to find any in old chronic cases.

The horse may suffer from either the acute or chronic form of glanders. In the acute the nasal mucous membrane is usually first involved. In the mucous membrane, particularly of the septum, nodules develop which break down, producing ulcers. The neighbouring lymphatic glands, the upper part of the respiratory tract and the lungs are also involved, similar nodules occurring in these situations and also in the internal organs, particularly the spleen. The animal generally dies within a month. In the chronic form of glanders (farcy) nodules appear in the subcutaneous tissue all over the body; ulceration of these frequently takes place. There is invariably considerable involvement of the superficial lymphatic vessels, which stand out as thickened cords, and also of the glands; secondary nodules may occur in the internal organs and the nasal mucous membrane. This chronic form of the disease may persist for years; in some cases cure results, but frequently the chronic disease becomes acute and the animal rapidly dies.

In man, glanders may be either acute or chronic. The primary lesion is generally in the skin, more rarely in the mucous membrane of the nose, mouth, or eye. There is a local, nodular swelling and inflammation of the lymphatic vessels, similar to that of the horse, and also a general skin eruption, at first papular, later pustular. Secondary foci appear in muscles, lungs, liver, or elsewhere. This acute form

is usually fatal in a few weeks. In the chronic type of the disease, nasal nodules which break down to form ulcers occur. Ulceration of the involved lymphatics also takes place. Complete recovery is rare.

The disease is spread from horse to horse chiefly by the infective nasal secretion and by the discharge from ulcers. Infection occurs through the skin or mucous membrane, by inhalation, or by ingestion. In man, infection is usually by the skin, chiefly in those dealing with horses.

Of the laboratory animals, the guinea-pig is the most susceptible, the rabbit being less so. Subcutaneous inoculation of the guinea-pig produces a local swelling which breaks down, forming a purulent ulcer. The lymphatic glands are involved, the spleen is usually enlarged and studded with nodules, and the other organs may show nodules. Following the intraperitoneal injection of a male guinea-pig with the bacilli, a purulent orchitis is set up which is almost diagnostic. Death generally occurs within one to two weeks.

Although the action of the glanders bacillus is largely local, leading to considerable tissue destruction, there is also a marked general effect which is due to the endotoxin produced by the organism, and it is to the action of this toxin that death is generally due.

During the course of the disease but little immunity is established and an animal, which has suffered from a chronic form of the disease, may die within a few days from an acute exacerbation. Antibodies such as agglutinins and complement fixing substances may be demonstrated in the serum of diseased animals, and may be used for the purpose of establishing a diagnosis, but these do not protect the animal against the bacillus. Just as in tuberculosis, a hypersensitiveness to the poisons of the bacilli is present and this is made use of in a diagnostic test for the disease in which a product known as mallein, prepared in a manner almost identical with that of old tuberculin, is employed.

The diagnosis of the disease, both in animals and in man, is a matter of great importance. In young lesions, the microscopical characteristics of the bacillus may be almost sufficient,

but no bacilli may be found in old lesions. If an unbroken nodule is available, direct culture should be attempted on glycerol-agar, potato and serum, but where other bacteria are present, one often fails to obtain a culture by plating.

Intraperitoneal injection of a male guinea-pig is a valuable method both for diagnosis and for the isolation of the bacilli for culture, since they are commonly found in a pure condition in the tunica vaginalis of the testicle. Certain indirect methods of diagnosis are also available, the most reliable of which are agglutination and complement fixation, using the serum of the affected animal. A simple and reliable test for the disease in horses is the subcutaneous or intradermal injection of mallein, which is used in a manner similar to tuberculin. Since the use of mallein is rather dangerous, it should not be employed for diagnosis in suspected cases of the disease in man.

Melioidosis, a rare tropical disease resembling glanders and affecting man, guinea-pig, rabbit, dog, cat and rat, is due to *Pf. whitmori*. This bacillus, which morphologically resembles *Past. pestis*, is motile and grows luxuriantly on agar. It actively ferments lactose, glucose and other carbohydrates, producing no gas. It does not produce indol.

## CHAPTER XL

### SPIROCHÆTES

THE spirochætes differ in many respects from the spirilla, the most important points being that the former usually possess a greater number of curves; during life they are not rigid, but are capable of bending; they do not possess flagella. The classification of this group of organisms is full of difficulties, and the controversy as to whether its members are to be considered as bacteria or as protozoa is not yet definitely settled. The name "spirochæte" has already been used for quite a different organism of the vegetable kingdom. It is, however, in such common employment for the parasites here considered that it would be rather pedantic to abandon its use completely. We will, therefore, refer to the organisms of the group as spirochætes (not Spirochætæ), but will use the terms "Spironema," "Treponema" and "Leptospira" in describing its individual members.

### THE SPIROCHÆTES OF THE RELAPSING FEVERS

In various parts of the world a type of fever, which is characterized by the alternation of periods of pyrexia with those in which the temperature is normal, is of fairly common occurrence. In these fevers, spirochætes are present in the blood stream during the pyrexial periods, and these spirochætes are all very similar in general appearances and effects, although they are to be regarded as distinct species.

**Spironema obermeieri.** *Borrelia recurrentis.*

Obermeier, 1873.

This spirochæte is very inconstant in length, varying from 7 to 30 $\mu$  or even longer: in thickness it is usually about

0.3 $\mu$ . Its curves may number from three or four up to as many as twenty in the very long forms. They are fairly sharp and well marked but, during life, do not rigidly hold their shape, the whole organism bending and straightening itself. It exhibits three forms of movement—a progression in either direction, a rotating or corkscrew motion, and bending. When a drop of infected blood is examined fresh the parasites are seen to move actively, pushing the blood cells from their path. During the height of the fever the number of spirochætes in the circulation may be enormous, several being visible in each microscopic field. The parasite stains faintly with watery solutions of the aniline dyes, but much better by Giemsa's method.

It has been cultivated in hydrocele fluid containing a piece of fresh rabbit tissue such as kidney.

In man, after an incubation period, there is a rapid rise of temperature and a pyrexial period of from five to seven days followed by crisis. After about a week another period of fever occurs which as suddenly gives place to normal temperature. In all, three or four paroxysms may occur, each being of less severity and shorter duration than the preceding. The mortality is low and the post-mortem findings are not noteworthy; an enlargement of the spleen and liver with gastric catarrh being of most common occurrence.

Monkeys are susceptible to the disease, and may be infected by subcutaneous injections of blood containing the spirochætes: in them relapses are not usual. Mice and rats are also capable of infection, but are less susceptible than monkeys and cannot be infected direct from man, only from the monkey.

The spirochæta appears in the blood in cases of the human disease shortly before the temperature begins to rise, and the number increases until fever is at its height. They remain numerous throughout the greater part of the period of pyrexia, but shortly before the crisis they disappear, and none can be found until the beginning of the relapse.

The nature of the immunity in this disease is of interest. At the crisis many spirochætes are phagocytosed, chiefly in the spleen, while others are agglutinated and very rapidly

fragment into small particles. The effect of antibodies is illustrated by the finding that, although spirochætes can live for weeks in blood removed early in the pyrexial period, they are quickly killed by blood taken at the time of crisis. Despite the presence of antibodies, a few spirochætes (possibly in the form of granules) survive and develop, causing the first relapse. The spirochætes of the first relapse are quite unharmed by the antibodies, because they have effected a complete change in serological type. Against them the patient develops new antibodies which cause a crisis and the spirochætes retort with a further change of type. This process is repeated, possibly several times, until presumably the spirochætes' powers of type transformation are exhausted. In each case the antibodies are very specific and are quite inactive against a later relapse strain. Such rapid changes in serological types render efforts at serological identification of the spirochætes impossible.

This variety of relapsing fever is of most common occurrence in Europe. The organism is conveyed by the body louse and, less frequently, by the bed bug. Infection may be caused by the feeding of the insect, but more commonly by rubbing into the wound the coelomic fluid of the crushed louse, in which spirochætes are present in large numbers.

Very similar spirochætes have been found to be the cause of relapsing fevers in other countries—*Spironema novyi* in America, *Spironema carteri* in India and *Spironema berberum* in Algiers. These organisms present no marked peculiarities sufficient to distinguish them from the *Spironema obermeieri* or from one another, but their pathogenic powers for animals are different.

Salvarsan and its derivatives are very useful in the treatment of all forms of relapsing fevers, but the development of arsenic-fast forms is to be guarded against by adequate dosage.

***Spironema duttoni.* *Borrelia duttoni.***

Dutton and Todd, 1905.

This organism is the cause of African relapsing fever or Tick fever. This disease is characterized by short periods of

pyrexia (two or three days), many relapses, and very low mortality. In microscopic appearance the parasite differs little from the *Sp. obermeieri*, but the number of parasites in the blood is much smaller. It is conveyed by the tick, more usually by its excretions than by biting, and the chief point of interest is that a tick may remain infective for periods of many months after a meal from an infected host. Further, the young of an infected tick are infective, even where it is certain that every possibility of fresh infection has been excluded. Since spirochætal forms can only be found for a limited period after a meal, it is probable that certain minute chromatin granules, which have been described as making their way through the tissues of the tick and appearing within the ova, are a stage in the development of the organism. This spironema readily infects a wide range of animals—monkey, horse, goat, sheep, dog, rabbit, guinea-pig, rat, and mouse.

## THE SPIROCHÆTES OF SYPHILIS AND YAWS

### *Treponema pallidum*.

Schaudinn and Hofmann, 1905.

This organism, which is most usually about  $8\mu$  in length, may measure from 3 to  $15\mu$ , and is extremely slender, rarely exceeding  $0.25\mu$  in thickness. It is characterized by sharp, deep, regular curves, the number of which depends on the length of the individual. The size of each curve is fairly constant, a distance of about  $1\mu$  separating the crest of one from that of the next, while the depth of the curve is from 1 to  $1.5\mu$ . The parasite tapers to each end, and terminates in a fine thread, which is not a flagellum. During life it is fairly actively motile, showing progression in either direction, rapid rotation around its long axis, expansion and contraction of its coils, and also slight flexion of the whole body.

It is alleged that this treponema has been cultivated by Noguchi's technique in a semi-solid mixture of agar and hydrocele fluid containing a piece of sterile rabbit's tissue, either kidney or testicle. Strictly anaerobic conditions and

body temperature are necessary for its growth which, in this medium, causes a faint haze spreading from the central inoculation tract, no other changes being produced.

The organism's powers of resistance are very slight, drying or moderate heating (55° C.) being rapidly fatal, but cold is well borne.

The treponema stains only with great difficulty (from which fact it derives its specific name, "pallidum"), the ordinary aniline dyes being without effect. Special stains (Giemsa, Fontana, or Levaditi) or the Indian ink method may be used, but in the case of fresh material the best results are obtained by examining the living organism unstained. On account of its low refractivity, the usual method of illumination does not reveal it, and indirect lighting, by the use of the dark field condenser, must be employed.

It is unnecessary to describe here the many clinical manifestations of syphilis. The *Treponema pallidum* has been found

in every lesion characteristic of the disease, even in such conditions as general paralysis and locomotor ataxia, which were formerly referred to as para-syphilitic. It occurs in greatest numbers, however, in the primary lesion, the chancre, and its identification in that situation is the earliest and simplest method of diagnosing the disease. The treponema may usually be found without difficulty in secondary lesions, and Warthin has shown that in gummata and other tertiary lesions prolonged search may reveal large numbers.

The disease is most commonly acquired in sexual intercourse, but non-venereal infections also occur. It is unlikely that the organism can penetrate the unbroken skin or mucous

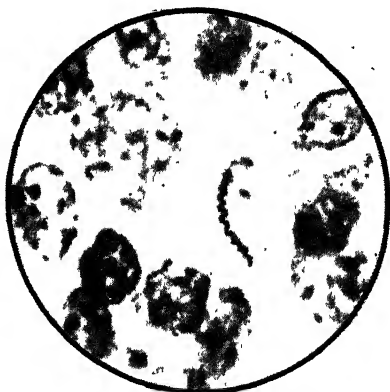


FIG. 67.—*TREPONEMA PALLIDUM* FROM TONSIL. WARTHIN'S STAIN ( $\times 1200$ ).



membrane, but contact of the slightest break in the surface with material containing the organism may be sufficient to cause infection. The protection afforded to those indulging in promiscuous sexual intercourse by the use of soap and water, with or without an antiseptic, shortly after connection, is undoubted. A most important type of innocent infection is that seen in congenital syphilis, the disease being acquired *in utero* from an infected mother, very rarely, if ever, from the father in the absence of syphilis in the mother.

The majority of animals are completely refractory to

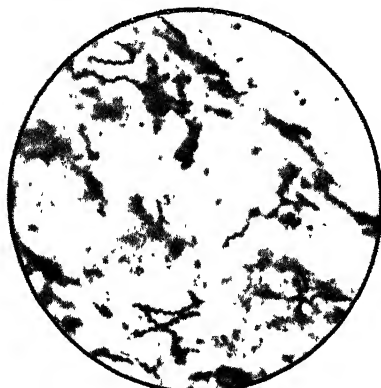


FIG. 68.—*TREPONEMA PALLIDUM* IN LYMPH GLAND. WARTHIN'S STAIN ( $\times 800$ ).

infection by the *Treponema pallidum*, and only in the higher apes, particularly the chimpanzee, is a disease similar to human syphilis produced. In order to infect this animal, scarification of skin or mucous membrane is essential, since the subcutaneous, intravenous, or intraperitoneal routes are ineffective. The most successful situations are on the eyebrows or the genitals. After an incubation

period of about a month, a local lesion, exactly comparable to the human chancre, develops. The drainage glands become enlarged and inflamed. After a further period of somewhat longer duration, secondary lesions develop in the skin and mucous membranes. Tertiary lesions have never been observed in these animals. Just as in the case of man, the blood serum of an infected ape shows a positive Wassermann reaction. The rabbit is, to a certain extent, also susceptible to infection, and by inoculating material containing spirochætes into the anterior chamber of the eye, a syphilitic iritis is established. Scarification and infection of the cornea leads to the production of keratitis. Injection of spirochætes into

the testis of a rabbit causes a severe orchitis, in which large numbers of the organisms may be found. The presence of a spirochæte (*Tr. cuniculi*), morphologically almost identical with *Tr. pallidum*, in naturally occurring lesions in rabbits, is to be borne in mind in connection with experimental work on syphilis in these animals. When *Tr. pallidum* is inoculated into a mouse, no sign of disease is produced, but the treponemata may be recovered from its glands, spleen, and brain several months afterwards.

Syphilis is essentially a chronic disease which is fatal only

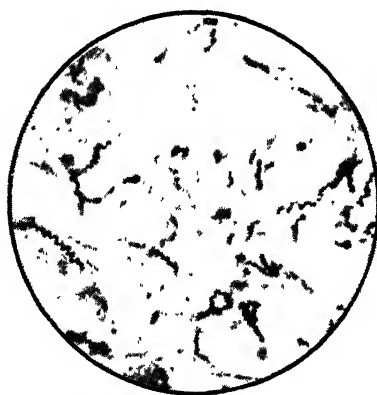


FIG. 69.—*TREPONEMA PALLIDUM* IN  
SECTION OF UTERUS ( $\times 950$ ).

after the lapse of a considerable time, usually many years, and then, as it were, only by accident such as the involvement of some essential organ or tissue. The spirochætes are disseminated throughout the body by the blood stream but only produce effects in a limited number of places at one time. These local effects may be considerable, but constitutional disturbances are very slight. It is probable

that syphilis is never a self-curing disease, but even under natural conditions, and without treatment, the syphilitic may have periods of intermission when no signs or symptoms of the disease are apparent. In a longer or shorter time, however, the latent spirochætes will again become active and produce definitely syphilitic lesions.

The problems of immunity in syphilis are full of difficulties. The disease, as has been said above, is not self-healing, that is, the presence of spirochætes in the human body does not lead to the development of anti-substances which are capable of destroying them. Nevertheless it is found that a man suffering from syphilis cannot again be infected with the disease, or

at least does not develop a primary lesion, but the syphilitic who has been cured may be again infected ; indeed, a definite re-infection is regarded as one of the strongest proofs of complete cure. In the case of animals also, it is true that re-infection is impossible during the disease, the only exception to the rule being in infections of the eye of the rabbit. The condition here is very strictly localized, and it is possible to infect the second eye while the first inoculated still shows signs of active syphilis.

As a result of immunization of certain animals with cultures, agglutinins and bactericidal substances are produced. These, however, are active only against strains of spirochætes which have been cultured in artificial medium. Freshly isolated spirochætes are completely resistant against such sera. For this and other reasons, many bacteriologists are doubtful if the organisms cultivated are really *Treponema pallidum*.

The Wassermann reaction, which is described in detail elsewhere, provides a very reliable method of diagnosing syphilis. The newer flocculation tests, of which the Kahn reaction is an excellent example, are in many respects superior to the Wassermann reaction, since standardization can be more thoroughly carried out. The Wassermann and Kahn reactions do not become positive until some weeks after the appearance of the chancre: in the absence of treatment they become stronger, and are positive in practically every case of secondary syphilis and in the majority of tertiary cases. In a high proportion of late syphilitic conditions of the central nervous system, they are positive in the blood or in the cerebro-spinal fluid, or in both. They are positive in the case of the majority of children presenting symptoms of congenital syphilis. As has been said before, remissions occur in the disease, and during these periods, when no evidence of active syphilis may be detectable, the reactions may become negative only to revert to positive when lesions reappear.

Despite the proved reliability of the tests, a diagnosis of syphilis should never be made on the result of a single test in the absence of clinical manifestations of the disease. Where a positive result is unexpected, the test should be repeated.

Apart from yaws, it is quite exceptional to get a Wassermann result of + or stronger or a Kahn result of ++ or stronger in the absence of syphilis. Doubtful reactions (Wassermann  $\pm$  or Kahn +) may occasionally occur where syphilis is almost certainly absent. Where the clinical evidence is suspicious, in an untreated case, and the result of the tests negative or doubtful, a "provocative dose" of salvarsan or its derivatives may be given and a test made one week later. This procedure will sometimes produce a definitely positive result. During treatment undertaken in the early stages of the disease the reactions soon become weaker (the Kahn remaining positive longer than the Wassermann in most cases) and ultimately negative; but this is not a proof of cure, for if an interval without any treatment is allowed to supervene, the reaction will again become positive and symptoms will reappear. Treatment should be continued for some time after a negative result has been obtained, and great caution observed before a true cure is claimed. Sufficient time has not yet elapsed since the use of salvarsan compounds became widespread, but, so far as we can judge, a patient in the primary or secondary stages who has received a full course of treatment and whose blood has given a negative reaction at intervals of three months over a period of two years is almost certainly cured, although such a good authority as Warthin was doubtful if the disease was ever really cured. In old standing cases, which have had no treatment or insufficient treatment, it may be impossible to reduce the reactions to negative. We cannot be dogmatic as regards these persistently positive reactions, but it may be that such results do not, of necessity, mean a continued presence of the spirochætes in the body.

For the diagnosis of the disease, the best method is the discovery of the organism. If a chancre is present, it should be well washed with saline to free it from contaminating organisms, and should then be vigorously rubbed with a piece of gauze. This operation is rather painful, but less so than the scarification usually recommended. The effect is to promote a flow of serum which may at first be bloody but later becomes clear. In this serum the spirochætes are usually present in

considerable numbers. They are best looked for with the dark field condenser and are recognized by their extreme thinness, their sharp, regular curves and their characteristic movements. In the case of genital sores, other spirochaetes may be present (*Spirochaeta refringens*, *Spirochaeta genitalis*), but these present little difficulty, as they are, for the most part, thicker with coarser and less regular curves than the *Treponema pallidum*. In lesions about the mouth, however, certain of the saprophytic mouth spirochaetes, particularly *Treponema microdentium*, which very closely resembles *Treponema pallidum*, may cause great difficulty in making a diagnosis.

In the absence of a dark field condenser, the organisms may be stained, preferably by Fontana's method, or preparations may be made by the Indian ink method. Examination in the living condition is, however, always better. Where tissue can be obtained for sections, Levaditi's or Warthin's method of staining, which are too complicated to be described here, give the best results.

In the secondary stage, where material can be obtained from a skin lesion, the organism may be searched for, but more usually the diagnosis is confirmed by the Wassermann or one of the other serological tests. Where a full positive result is obtained on more than one occasion, and where yaws can be excluded, syphilis is almost certainly present. A confirmatory test, not strictly bacteriological, is the rapid disappearance of skin lesions when one of the salvarsan compounds is administered.

### ***Treponema pertenue*.**

Castellani, 1905.

This organism is almost identical in microscopical appearance with *Treponema pallidum* and the disease which it produces—Yaws or Framboesia—is, in many respects, very similar to syphilis. It is a general disease which is characterized by the occurrence of papular eruptions in various parts of the body. By inoculation of monkeys with material containing the organism, it is possible to produce a general disease, closely resembling the disease in man.

Both in animals and in man, as a result of yaws, the serum gives a positive Wassermann reaction. Yaws is regarded by many as a modified form of syphilis; but it must be looked upon as a distinct, though closely allied, condition, since the two diseases may coexist in man, and since an animal infected with one is still capable of infection with the other.

## THE LEPTOSPIRÆ.

### *Leptospira icterohæmorrhagiæ.*

Inado and Ido, 1914.

This organism measures from 6 to  $12\mu$  in length and is about  $0.25\mu$  in thickness. It exhibits very fine, close-set curves ( $0.5\mu$  between crests and  $0.3\mu$  deep) which, unless preparations are carefully made, may not be seen, and also a coarse waviness. Its greatest point of distinction from the other spirochætes is that one or both ends are sharply curved, forming terminal hooks. In motility it resembles other spirochætes.

It has been cultivated in Noguchi's semi-fluid serum-agar, but the presence of rabbit's tissue is not essential. It is microaerophilic rather than anaerobic and, if cultures are made in deep tubes, it is unnecessary to cover the surface of the medium with oil. The optimum temperature of growth is from  $25^{\circ}$  to  $30^{\circ}$  C.

Weil's disease, or epidemic jaundice, has been observed in many parts of the world. It is characterized by the occurrence of pyrexia, gastro-intestinal symptoms, jaundice, hæmorrhages, either into the tissues or from mucous membranes, and albuminuria. The mortality may be high (35 per cent.), as in Japanese epidemics, low (2-3 per cent.), as occurred in soldiers during the European War, or intermediate (10 per cent.) as with cases occurring in Holland.

The parasites are present in the blood stream during the first few days of the disease, but as the number is small, direct examination may fail to reveal their presence, which is most easily detected by animal inoculation or by direct culture. When jaundice becomes marked, the organisms can no longer

be found in the blood but are present, often in considerable numbers, in the internal organs, particularly in the liver, kidney and adrenals. Later, they disappear from the organs, but may continue to be excreted in the urine for several weeks after convalescence.

The guinea-pig may be infected with most strains as a result of either intraperitoneal inoculation or scarification of the skin. The animal develops jaundice and death occurs in about ten days after inoculation. Hæmorrhages are found subcutaneously, and in the lungs and intestine, and the spleen

is enlarged. The organisms are numerous in the kidneys, adrenals, and liver.



FIG. 70.—LEPTOSPIRA ICTEROHÆMORRHAGIÆ IN SECTION OF KIDNEY OF AN INFECTED GUINEA-PIG ( $\times 1200$ ).

The nature of the immunity developed in man against the parasite is shown by the finding of agglutinins and bacteriolysins in the serum of convalescents. It is rather difficult to explain the continued presence of the leptospiræ in the urine and in the kidney in view of the existence in the blood of these antibodies.

As a result of the immunization of animals, antiserum can be obtained which appears to be of considerable benefit therapeutically.

The chief source of infection of man is the rat, which acts as a reservoir of the leptospiræ, the organisms lodging in the kidneys and being excreted in the urine. A high proportion of wild rats in all countries harbour the parasite. Human infection may occur through eating food contaminated with rat urine, but an indirect method of spread is more common. The leptospiræ can survive in water for a considerable time: it is even possible that, under suitable conditions, they may

grow in it. Human beings may become infected by immersion in such contaminated water, the organisms entering the body by the mouth or through the skin or mucous membranes of the eyes or nose. The combination of rats and moisture explains the occurrence of cases in sewer workers, miners, and those employed in cleaning fish and in preparing tripe. Cuts and abrasions of the skin probably facilitate the penetration of leptospiræ into the tissues. *Lepto. icterohæmorrhagiæ* is not the only leptospira found in water. Even pure water not uncommonly contains *Lepto. biflexa* which differs from *Lepto. icterohæmorrhagiæ* chiefly in being devoid of pathogenicity for animals.

Another leptospira, *Lepto. canicola*, causes uræmia in dogs or may be excreted in the urine of healthy dogs which are carriers. In man, the disease caused by this organism is similar to Weil's disease but is usually milder and jaundice is not such a marked feature.

Diagnosis of the disease is usually made by the intra-peritoneal inoculation of a guinea-pig with the patient's blood in the first few days, or, later, with the centrifuged deposit of the patient's urine. Since some human strains have little pathogenicity for animals, direct culture from the same materials should be attempted. Microscopic examination of the deposit from the urine with the dark field condenser is often sufficient, but care must be exercised since other spirochætes may be present. The characteristic hooked ends of the leptospiræ are, however, of great diagnostic importance. Serological methods of diagnosis are also available. The serum of a patient agglutinates *Leptospira icterohæmorrhagiæ* either living, after 2 hours at 32° C., or in formalized culture, after from 4 to 12 hours at room temperature. The test is usually positive on the sixth day of the disease and, by the twentieth day, the titre of the serum may be  $\frac{1}{100000}$ . An alternative method is the adhesion test in which, after the fresh unheated serum of the patient has been kept in contact with cultures of the organism and of *Bact. coli* at 37° C. for 30 minutes, it is found that the bacilli are firmly adherent to the leptospiræ.



It is stated that other varieties of leptospiræ are causative agents in a number of Oriental diseases, such as Seven-day Fever, which is due to *Leptospira hebdomadis*, the organism being spread by the field mouse.

### OTHER SPIROCHÆTES.

In Vincent's angina, a condition of the throat, pharynx, or mouth, in which a false membrane with ulceration occurs, a



FIG. 71.—*SPIRONEMA VINCENTI* AND *F. FUSIFORMIS* FROM THROAT OF A CASE OF VINCENT'S ANGINA ( $\times 950$ ).

very characteristic microscopical picture is obtained by staining films made from the lesion with gentian violet or carbol-fuchsin.

Large numbers of bacilli, the *Fusiformis fusiformis* (*F. dentium*), are seen associated with very many spirochætes, *Spironema vincenti* (*Borrelia vincenti*). The bacilli, which

measure from 3 to  $10\mu$  in length by about  $0.7\mu$  in the centre, taper sharply

towards either end. They are usually straight or slightly curved. They stain with some difficulty and carbol-fuchsin gives the best results. Most characteristically the stain is not taken uniformly, the bacilli presenting a striped or beaded appearance, a very common form having an unstained central bar reminiscent of Hofmann's bacillus. The spirochætes are rather longer than the bacilli, but are very thin and also stain with difficulty. They have a variable number of loose, irregular curves. The practically constant presence in large numbers of the two organisms in clinical Vincent's angina is suggestive of their ætiological importance; but exactly similar bacilli and spirochætes may be found in many normal mouths, or in the neighbourhood of teeth which are carious

or around which pyorrhœa alveolaris is present, but here they are rarely so numerous as in Vincent's angina.

Both the bacilli and the spirochætes have been cultivated under anaerobic conditions, and although it has been claimed that the bacilli develop into the spirochætes, this has not been substantiated. They are almost certainly distinct organisms which thrive symbiotically, and which of the two, if either, is to be regarded as causative, or whether the combination is responsible for the condition, is as yet undecided. The rapid cure, so frequently effected by the salvarsan compounds, suggests the spirochæte as probably of greater importance. A similar combination of the two organisms is also found in certain cases of tropical ulcer.

Many other types of spirochætes have been described, but the majority of these appear to be either normal saprophytes or to have but a low grade of virulence. Many of these are present about the mouth (*Sp. buccalis*, *Sp. macrodentium*, and

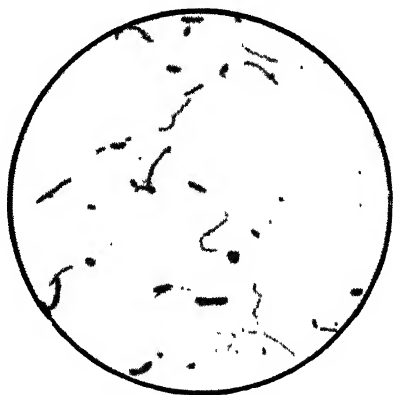


FIG. 72.—SPIROCHÆTES AND OTHER BACTERIA FROM TOOTH (FONTANA STAIN) ( $\times 950$ ).

*Sp. microdentium*), particularly in the neighbourhood of decaying teeth. They also occur in gangrenous conditions of other parts of the body.

It is convenient to consider here Rat-bite fever, although the causative organism, *Spirillum minus* (*Spironema morsus muris*) is more correctly grouped with the spirilla than with the spirochætes. This spirillum, which is commonly present in rats in various parts of the world, measures from 2 to 5  $\mu$  in length, and has close, regular turns. It is actively motile owing to the presence of flagella at both ends. The disease is usually produced by the bite of a rat. After some weeks,

the site of the wound, which may have healed, becomes inflamed and breaks down; the drainage glands become swollen, there is pyrexia, sudden in onset and remittent in type, and a purple skin rash. The spirilla are present in the blood and are few in numbers. The easiest method of diagnosis is to inject blood into a mouse, in the blood of which spirilla may be found after ten days. A single injection of neo-salvarsan usually effects a cure.

## CHAPTER XLI

### TOXIN-PRODUCING ANAEROBIC BACILLI

#### ***Clostridium tetani.* (*B. tetani.*)**

Nicolaier, 1884.

THE tetanus bacillus measures from 2 to  $5\mu$  in length by  $0.3$  to  $0.7\mu$  in thickness. It is a straight, round-ended bacillus which is most commonly found singly or in pairs, but long chains of bacilli occur in cultures. It is actively motile, has no capsule, and produces spores. These spores are produced both in the bodies of animals and in culture. They are terminal and spherical or oval, measuring from 1 to  $1.5\mu$  in diameter, and the bacillus with its spore closely resembles a drum-stick in appearance. The bacillus stains readily and is Gram positive.

*Cl. tetani* is an obligatory anaerobe, which can grow *in vacuo* or in an atmosphere of hydrogen or nitrogen, but not in carbon dioxide. Its optimum temperature of cultivation is that of the

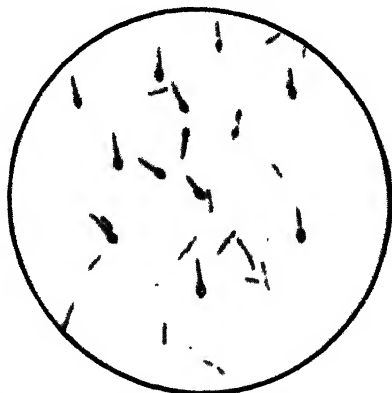


FIG. 73.—*CL. TETANI* FROM AGAR CULTURE ( $\times 950$ ).

body, but growth goes on slowly at  $20^{\circ}\text{C}$ . In broth, a fine turbidity, with later a deposit at the bottom of the tube, is produced. Meat broth is unaltered, no digestion of the meat and no discoloration being detectable. In an agar stab, growth occurs along the track of the wire, and from this very

delicate offshoots pass into the medium. On moist agar, the growth is in the form of a thin film which spreads rapidly over the surface. Hæmolysis is produced on blood agar. Gelatin is liquefied and there is some digestion of egg white. Indol is produced and glucose slightly fermented.

Spores form in from 24 to 48 hours at body temperature, but much more slowly at air temperature. In the wet state, the spores withstand a temperature of 80° C. for several hours, but are usually destroyed in less than a quarter of an hour by boiling. Like other spores, they are much more resistant against heat in the dry state. Kept dry, in the dark and in the cold, they have been found to be still viable after eighteen years. They resist antiseptics well, and may survive ten hours' exposure to 5 per cent. phenol; 1 per cent. silver nitrate solution, however, kills them in one minute, and chlorine is also active against them.

The tetanus bacillus is widely distributed in the superficial layers of the soil in all parts of the world, but is more often found in richly cultivated land than in virgin soil. It is commonly present in the fæces of herbivora, particularly of horses and, in some countries, in the fæces of man. The intestine is probably its normal habitat, where it lives as a harmless saprophyte.

The disease which it produces, tetanus, occurs most frequently in man and in the horse, but the majority of mammals are susceptible to artificial inoculation. Birds and reptiles are almost or completely insusceptible. In man the disease is usually the outcome of a wound, particularly of a deep stab or penetrating wound or one which is much lacerated and contused. The presence of dirt, of foreign bodies or of other bacteria, is almost to be considered as requisite for the production of tetanus. A number of cases of tetanus have been traced to the use of improperly prepared catgut in operations. Mackie states that a watery solution of iodine and hydrogen peroxide are the best disinfectants to use in the sterilization of catgut. The commonly employed biniodide solution and alcohol are quite useless. The type of the disease which was formerly known as "idiopathic" was

almost certainly due to a slight wound, not then regarded as having any connection with the disease. The period of incubation may be as short as a few days or may be months. The bacilli or their spores may lie dormant in the tissue until some later injury, such as operative interference, gives them a suitable nidus for their further development. The organism has been isolated from the site of an old wound more than two years after its infliction. Another form of the disease, that occurring in new-born infants, is due to infection of the umbilical cord. The bacilli inflict very little local injury. Pus may be present in small amount, but this is due chiefly to the presence of other bacteria. There is no great multiplication of the bacilli and practically no invasion of tissues, although they have, on a few occasions, been isolated from the spleen. The effects which they produce on the body, the terrible tonic spasms of muscles, are due entirely to the exotoxin produced by them. This toxin is taken up by the end organs of the motor nerves and passes up their axis cylinders to the central nervous system. Some passes also to the blood stream, but can only reach the central nervous system by way of the peripheral nerves. The spasms are central in origin, and are due to the poisonous action of the toxin on the nerve cells in the cord. The affinity possessed by nerve cells for toxin is shown by the fact that, by mixing toxic broth with brain substance, the former is completely deprived of its poisonous properties. The disease is very fatal unless modified by the previous or almost coincident administration of antitoxic serum. The shorter the period of incubation, the worse is the prognosis. The post-mortem changes are very slight, some congestion of the internal organs and of the central nervous system, with a degeneration of the ganglion cells, being all that can be detected.

Inoculation of laboratory animals with material from a human case reproduces the disease. Subcutaneous injection first causes local spasms in the neighbourhood of the site of injection. These soon become general and death occurs within a few days. It is found that no matter how large a dose is given, an incubation period, at least eight hours in the

mouse, elapses before symptoms appear. It is probable that this represents, in part at least, the time necessary for the toxin to travel along the nerves. The inoculation of one of the smaller animals either with cultures of the bacillus or with soil or other material containing bacilli invariably causes typical tetanus to develop in the animal. If, however, the spores are carefully freed from toxin by washing and injected suspended in saline, or if the toxin present in the culture is destroyed by heating to 80° C., the animal frequently escapes. The addition of other bacteria, foreign bodies such as a splinter of wood or earth, lactic acid, calcium chloride, quinine, or of their own toxin is sufficient to cause the disease to appear. The explanation is that if spores are injected alone, some are phagocytosed and so carried to various parts of the body, where they lie dormant, as too much oxygen is present to permit them to vegetate. The tissue debilitants mentioned cause either local necrosis or, at least, interfere with normal blood supply, so that the oxygen tension of the tissues is lowered to a level which allows of development of the bacilli. Where spores are injected in one locality in an animal's body and a tissue debilitant in another, the bacilli grow only at the latter site. This experimental work explains the well-known fact that a badly lacerated and contused wound, or one contaminated with dirt, is a more likely precursor of tetanus than is a clean wound inflicted with a sharp instrument.

Toxin is produced not only in the animal's body but also in culture medium. A suitable broth is essential, as also are anaerobic conditions. The maximum yield of toxin is found at the end of from 7 to 14 days' incubation, although little growth occurs after 48 hours. The toxic broth is freed from bacilli by filtration through a Berkefeld or Chamberland filter, and, after the addition of 0.5 per cent. phenol, may be preserved in the dark at a low temperature and protected from oxygen. The reverse of these conditions (the presence of oxygen, high temperature and bright light) causes a rapid fall in the toxicity of the broth. A temperature of 65° C. completely destroys the toxin in less than one hour. The toxin may be concentrated by precipitation, the

broth being saturated with ammonium sulphate and the precipitate freed from the salt by dialysis. The dry solid obtained by evaporation *in vacuo* is not pure toxin, but it is very rich in toxin, and its strength is retained for long periods if kept dry and in the cold. Tetanus toxin is a most powerful poison, as small an amount as 0·000005 c.c. of the filtrate or 0·000001 gm. of the dried substance being sufficient to kill a mouse. It has been calculated that one quarter of a milligram would probably be fatal for a man.

The toxin takes effect on animals when administered subcutaneously, intramuscularly, or intravenously. It is, however, without effect when given by the mouth, some being destroyed by gastric and intestinal secretions and by the action of intestinal bacteria and some being excreted unchanged with the fæces. In a toxic filtrate two distinct poisonous substances have been found to be present ; the first, (tetanospasmin), acts as an exciter of the motor cells in the cord, pons, and medulla and is the cause of the spasms. The other, (tetanolysin), causes hæmolysis of red blood cells and is probably not of any great importance in the disease.

By the immunization of animals (usually horses) an anti-toxic serum can be prepared. The animal receives at first a very small dose of toxin, or of toxin converted into toxoid by the action of formalin. On subsequent occasions, the dose is increased until no harm results from very large injections of potent toxin. The serum is collected as in the preparation of diphtheria antitoxin. A satisfactory serum contains from 300 to 1,500 units in each cubic centimetre.

The great success of the serum treatment of diphtheria led to corresponding hopes in connection with tetanus antitoxin, but these expectations were not realized when the serum was tested. For this there are many explanations: the disease presents no striking local lesion which allows of its diagnosis before symptoms of intoxication are present ; the toxin has a greater affinity for nerve cells than for its antitoxin ; the nerve cells are more susceptible to damage than the sturdier muscle or gland cells attacked by diphtheria toxin ; diphtheria toxin circulates in the blood where it can be acted upon by



antitoxin injected, while tetanus toxin is removed from the circulation by the nerve end organs, whence it passes by way of the nerves to the nerve cells, and in this route it is but slightly exposed to antitoxic action. Animal experiments have shown that, if the amount of antitoxin be found which is sufficient to protect against a given dose of toxin when the two are administered together, the antitoxin must be enormously increased when even a few minutes supervene between the administration of the toxin and the antitoxin. The moral is that antitoxin, to be really successful, should be given before symptoms appear; that is, that a prophylactic injection should be made in the case of every dirty wound, just as to-day antidiphtheritic serum is given in every suspicious throat case. The prophylactic dose should be 2,000 to 3,000 units.

The experience of the European War demonstrated the value of prophylactic administration of antitoxin in preventing the development of tetanus or in causing a milder form of the disease if it did subsequently occur. In every suspicious wound, serum should be given twice or thrice at weekly intervals. Without serum the mortality from tetanus is 80 to 85 per cent., and although even with the free use of antitoxin declared tetanus is a very fatal disease, nevertheless serum should be given. On account of its slow rate of absorption, the subcutaneous or intramuscular routes are unsuitable, except to act as reservoirs to maintain the concentration of antitoxin in the body. Every moment is of importance, and large amounts of serum should be given both intrathecally and intravenously. In all two hundred thousand units to even more may be administered and, although death frequently occurs despite this, the successes have been sufficiently numerous and, in a number of cases, striking to warrant the fullest use of antitoxin, even in apparently hopeless cases.

A satisfactory degree of active immunity may be produced, in soldiers or others who run a considerable risk of contracting tetanus, by two injections of tetanus toxoid. For many years pit ponies, which are prone to contract tetanus, have been actively immunized in this way.

Diagnosis of the declared disease is usually a clinical matter, but the finding of typical drum-stick bacilli in a wound is suggestive, although there are many other bacilli similar to *Cl. tetani*. The organism should be isolated in pure culture which will be facilitated if the material be heated to 80° C. for ten minutes to destroy non-sporing bacteria and inoculated into the condensation water of an agar slope. After twenty-four hours incubation anaerobically a pure culture may frequently be obtained by picking from the filamentous edge of growth which may have spread a considerable distance up the agar surface. The identification is completed if the isolated organism causes tetanus in animals inoculated with it. Inoculation of animals with impure cultures is not always successful, as the other bacteria present may destroy tetanus toxin.

Since there are a number of serological types of tetanus bacilli (all of which produce the same toxin) agglutination tests are useless for the recognition of the organism.

***Clostridium botulinum.* (*B. botulinus*.)**

Van Ermengem, 1896.

*Cl. botulinum* is a large, straight, round-ended, slightly motile bacillus, measuring from 4 to 6 $\mu$  by 0.9 to 1.2 $\mu$ , which is found singly or, more rarely, in short chains. The bacillus stains readily and is Gram positive. It produces oval spores which are only slightly thicker than the bacilli and are situated at or near the ends.

The organism is strictly anaerobic, and grows fairly easily on ordinary media of neutral or slightly alkaline reaction; the presence of glucose greatly assists growth. The optimum temperature of cultivation is about 25° C. On solid medium, the colonies are yellow, translucent and coarsely granular, and later become more opaque and brownish. On blood-agar, it is hæmolytic. In stab-cultures in gelatin or agar containing glucose, the medium is cracked and split by the evolution of gas. Gelatin is rapidly liquefied, but strains which produce toxin have been described in which the power of liquefying gelatin is absent. Glucose and maltose are

always fermented: some strains ferment saccharose: none ferment lactose. In meat medium there is some digestion and darkening of the meat. All cultures have a rather unpleasant rancid odour, owing to the production of butyric acid.

The resistance of the spores to heating is variable. The majority of bacteriologists have found them much less resistant than the spores of other bacteria, being usually killed in one hour at 85° C. and rapidly at 100° C. Some highly resistant spores have, however, been described which survived boiling for several hours and 120° C. for 20 minutes. This inconsistency may, in part at least, be due to the fact that acidity of the medium has a marked effect in lowering the lethal temperature, as has also the presence of salt.

The natural habitat of the organism is not known with certainty, but it is probable that it carries on a saprophytic existence on vegetable matter in the soil. Its importance to man is in its occurrence in various articles of diet in which it forms an active poison. The nature of the food is very varied, but practically all forms have one thing in common—they are preserved. The disease produced (botulism) derives its name from some outbreaks due to eating sausages, but ham (usually eaten raw), tinned meats, tinned fruit (apricots and pears), vegetables (asparagus, peas, beans and olives), fish and other foods have been incriminated.

The bacillus itself does not appear to be pathogenic, and multiplies little, if at all, in the alimentary canal. It can only rarely be isolated from the intestines of victims, and only in one or two cases has it been found in the spleen. The disease is due to the toxin which is formed by the growth of the bacilli in food. From what has been said before we can judge of the conditions suitable for the production of this toxin: a warm air temperature, absence of oxygen and suitable food material (not necessarily animal) are essentials, and these are frequently to be found in large sausages, smoked hams and tinned foods. The growth of the bacilli usually produces little or no change in the food, which is never apparently "bad," although a slightly rancid odour may be detectable.

Botulism differs from the other forms of food-poisoning in that gastro-intestinal symptoms are very slight. The action of the toxin is chiefly on the cranial nerve centres, and this is responsible for the most prominent symptoms, involvement of the eye muscles with protrusion of the eyeballs, ptosis, loss of accommodation, dilated pupils, as well as aphonia and dysphagia. Disturbances of the flow of saliva, either dryness of the mouth or salivation, are commonly present. Constipation and retention of urine are sometimes noted; fever is generally absent and consciousness is retained. Symptoms usually appear within 24 hours of eating the food and death occurs 4 to 8 days later. Death is due to involvement of the respiratory or, more rarely, of the cardiac nerve centres. Since the disease is due to the preformed poison, it is more comparable to acute poisoning than to one of the infectious diseases in which the causative organisms multiply in the body. The severity of the symptoms depends on the amount of food (and therefore toxin) consumed. This fact may explain the differences in the fatality rates in different outbreaks which average about 65 per cent.

The toxin may be prepared in artificial cultures, the most favourable medium being glucose broth. A filtrate of an eleven-day culture is very toxic for animals, and 0·0001 c.c. given subcutaneously may be fatal for a guinea-pig in 18 to 36 hours. The toxin is thermolabile, being completely destroyed in thirty minutes at 80° C., and rapidly deteriorates at air temperature when exposed to light and air. It is fatal to the majority of experimental animals, mice, guinea-pigs and cats especially, the rabbit being less susceptible. This toxin differs from those of *Cl. tetani* and *C. diphtheriæ* in that it is not destroyed by the gastric or intestinal fluids and it can be absorbed from the intestine, about three times as large a dose, however, being necessary to kill the animal when given in food as when injected subcutaneously.

In animals the striking symptoms are a general motor paralysis, protrusion of the eyeballs and dyspnoea, and death in from 24 to 48 hours. The post-mortem appearances in man and animals are not striking; there is a general

hyperæmia of the internal organs ; minute hæmorrhages and, in many cases, thrombosis are also observed.

An antitoxin has been prepared against the toxin, and it has been found that there are at least two distinct types of *Cl. botulinum*, causing botulism in man. These are morphologically and culturally indistinguishable. The toxins of these two (A and B) have the same action but their antitoxins differ, the antitoxin for one failing to neutralize the toxin of the other. The antitoxic serum is prepared in a manner similar to that used in the case of diphtheria or tetanus antitoxins. It is capable of protecting an animal against many fatal doses of toxin when administered previously or simultaneously, but not when symptoms have appeared. It has been used therapeutically in the disease in man in a few cases, but it is of doubtful value. It is conceivable, however, where a number of persons have partaken of the same food, and when one or more have developed symptoms of botulism, that the administration of the serum to those unaffected might have a protective action. In the treatment of a case of botulism, iodine or potassium iodide may be given by the mouth, since these substances are destructive of the toxin, and an olive oil enema should be administered in order to prevent absorption of the toxin from the intestine.

In addition to types A and B mentioned above, three other types (C, D and E) of *Cl. botulinum* have been described. These are responsible for a variety of diseases in animals but have not been recorded as causing botulism in man. They differ mainly in the toxins which they produce.

Diagnosis is usually made on clinical grounds and, from the character of the disease, presents little difficulty. Bacteriological confirmation is not generally possible by the isolation of the organism from the body, but cultures should be made from all suspected food. Glucose-broth and glucose-gelatin are the media of choice, and, by heating to 60° C. for half an hour, the food may be freed from the majority of non-sporing bacteria. The intraperitoneal inoculation of suspensions of suspected food into three mice, one of which was protected a few hours before by the antitoxin to the A type of toxin and

a second by the B antitoxin, may be sufficient to establish the diagnosis, giving, in addition, information as to the type of bacilli present.

Prophylaxis is chiefly a matter of the hygienic preparation of foodstuffs. Since the toxin which is the cause of the disease is thermolabile, the heating of any food, which may be suspected, to 100° C. for a few minutes immediately before use, will make it quite safe. Anaerobic conditions are of course most perfect in tinned and bottled foods, and these should be rejected if they are not in perfect condition, as judged both by the eye and by the nose. While a large number of cases have been due to tinned or bottled foods, it is right to point out that these have usually been home-prepared. Factory methods of sterilization are more accurately carried out than are those of the private house. Since botulism is a very rare condition, the possibility of its occurrence should not rule out of the dietary useful foods, of which many millions partake in safety for every one who suffers from the disease. If there is the slightest suspicion of the food, it should not be eaten. Death has resulted from eating half an olive.

## CHAPTER XLII

### ANAEROBIC BACTERIA OF WOUNDS

**Clostridium welchii.** (*B. perfringens*, *B. aerogenes capsulatus*,  
*B. enteritidis sporogenes*. *B. welchii*.)

Welch and Nuttall, 1892.

THIS organism is a large, Gram positive bacillus with square-cut or slightly rounded ends. Most characteristically it measures from 3 to 6 $\mu$  by 0.8 to 1.2 $\mu$ , but occasionally short, almost coccal, forms as well as filaments, are seen. It is usually found singly, or in pairs: chains are rare except



FIG. 74.—CL. WELCHII FROM AGAR  
CULTURE ( $\times 800$ ).

in old cultures. It is non-motile and possesses no flagella. Capsules are present in the tissues of the body, but are only to be found in cultures when the medium contains serum. The organism produces oval spores which are of smaller diameter than the bacillus and are situated most usually towards one end. Spores are not seen in the tissues or fluids of

the body and are not numerous in culture: none are produced in media containing any carbohydrate fermented by the bacillus.

*Cl. welchii* is a strict anaerobe. Growth is not abundant except in the presence of glucose or other utilizable carbohydrate. In meat broth there is some clouding, the meat

becomes slightly pinkish in colour, and gas is evolved abundantly. There is no evidence of digestion and the odour is sour, owing to the production of butyric acid, but not putrid. A very characteristic change is produced in whole-milk cultures. There is coagulation of casein into a clot, which floats in an almost clear whey. The clot and layer of cream are torn and riddled owing to the active evolution of gas. This "stormy fermentation" of milk is of importance in identifying the organism. Gelatin is liquefied. The organism is very feebly, if at all, proteolytic, but is actively saccharolytic, glucose, maltose, lactose, saccharose and other carbohydrates being fermented with vigorous gas evolution. The bacillus is actively hæmolytic. The body of a rabbit furnishes an excellent culture medium; the changes produced are rather characteristic and the method is good for the isolation of the organism. The test, known as the Welch-Nuttall test, is performed as follows: the culture or other fluid is injected intravenously into an ear vein and, after about five minutes, the animal is killed and the body left in the incubator for 12 to 18 hours. It will then be found distended with gas, bubbles of which will be found along the vessels and in the organs, particularly the liver. Films made from the site of one of these bubbles will show *Cl. welchii* and, by culture, the organism is found to be present, usually in pure condition.

The vegetative form of the bacillus has no special powers of resistance and the organism dies fairly rapidly in cultures in which glucose or other fermentable sugar is present, both owing to the absence of spores and to the acid produced. The spores are resistant and may withstand boiling for a few minutes.

The bacillus is widely distributed in nature, being found in the soil, in water, in milk and in the fæces of many species of animals, including man. Gas blisters and foamy organs, due



FIG. 75.—  
STORMY  
FERMEN-  
TATION OF  
MILK BY *CL.*  
*WELCHII*  
( $\times \frac{1}{2}$ ).



to its presence, are fairly commonly found at post-mortem examinations, but in the majority of cases the invasion of the tissues occurs after death. Although commonly present in the human intestine, it is there usually as a saprophyte; but there is some evidence in favour of its being a cause of enteritis, and it has been claimed that excessive numbers of the bacilli in the intestine cause certain forms of anæmia. In pernicious anæmia there is generally a great excess of the bacilli in the fæces, but this may be largely due to the condition of achlorhydria which permits the growth of the bacilli in the



FIG. 76.—*CL. WELCHII* IN MUSCLE  
( $\times 950$ ).

duodenum and even in the stomach. Its most important pathogenic rôle is in gas gangrene, a condition which occurs rather rarely in civil practice, but was common during the European War. Extensive wounds, in which there is great destruction of tissue and excessive contamination with soil, are those most likely to develop gas gangrene. *Cl. welchii* is not the only

organism causing the condition, but it is the one most commonly found. Rarely, however, does it occur alone, but is most usually accompanied by other bacteria, both aerobic and anaerobic. Among the latter one of the most important is the *Cl. sporogenes*, in the presence of which the virulence of *Cl. welchii* appears to be increased.

In gas gangrene due to *Cl. welchii* there is a rapidly spreading necrosis of tissue, almost always of muscle, together with considerable hæmorrhagic œdema and gas production. The muscles are pale, swollen and friable. The bacilli are found abundantly between the dead muscle fibres, but it is observed that the level of necrosis is in advance of the bacilli

and the gas which they produce. The condition may progress rapidly and death may occur within twenty-four hours of its onset. Many of the general symptoms associated with acute intestinal obstruction and general peritonitis are similar to those of gas gangrene, and it has been suggested that these are due to absorption of *Cl. welchii* toxin through the damaged mucous membrane. It is claimed that the use of *Cl. welchii* antitoxin is of value in such cases.

Guinea-pigs and mice are susceptible to the action of the bacillus, but rabbits show much greater powers of resistance. In the former animals, injection of a virulent strain produces a condition very similar to that found in man. The virulence of different strains is very dissimilar; some appear to be almost devoid of pathogenic properties, but others are exceedingly virulent.

*Cl. welchii* produces a soluble toxin during the early period of growth of the bacilli in artificial culture: it reaches its maximum strength in about twenty-four hours and then rapidly deteriorates. The toxin, which is destroyed at 70° C. in half an hour, produces its effects very rapidly in the body of an inoculated animal, and death may take place in two or three hours. The post-mortem appearances are very similar to those caused by the injection of the living organism itself, except for the absence of gas. The toxin is not produced in such concentration as that of *Cl. tetani*, but the minimum lethal dose of the filtered broth for a guinea-pig may be 0·25 c.c. Its effects are chiefly local, including cedema, necrosis and hæmolysis. An antitoxin has been prepared, and is found useful in the treatment of cases of gas gangrene due to this bacillus. In media containing glucose, another poisonous substance is produced. This resembles histamine in its mode of action. It is thermostable and non-antigenic, that is, an antitoxin cannot be developed against it.

The description of *Cl. welchii* given above refers mainly to the A type of the bacillus, the only one of known pathogenicity for man. Three other types, B, C, and D, have been described. They produce, in varying amounts, three other toxins. These types are responsible for a number of diseases in lower animals.

**Clostridium septicum.** (*Vibrio septicum*.) *Clostridium oedematis-maligni*.

Pasteur and Joubert, 1877.

Cl. septicum is a straight or slightly curved bacillus, measuring from 3 to 8 $\mu$  by 0.8 to 1 $\mu$ , but very much longer forms are frequently seen, both in the tissues and in culture. It is found lying singly, in pairs, or in chains, and occasionally in long filaments in which it is doubtful whether

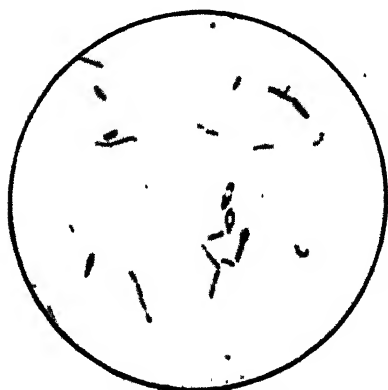


FIG. 77.—CL. SEPTIQUE FROM BROTH CULTURE ( $\times 850$ ).

subdivisions into bacilli are present. It is feebly motile, but under aerobic conditions is non-motile. No capsules have been observed. The organism stains easily and young forms are Gram positive, but in older cultures it may be Gram negative. Oval spores, thicker than the bacilli, are produced and are situated either centrally or sub-terminally. Spores are rarely produced in the living

body but are found in cultures, especially in a medium containing body fluids and with a minimum of utilizable carbohydrate. Two kinds of involution forms are observed; the first of these consists of the long filaments, which are most regularly found on the surface of the liver and other abdominal organs when the peritoneal cavity has been invaded in animals experimentally inoculated; in the other, known as the navicular or citron form, the bacilli are short, thick in the centre and tapering towards the ends, or almost oval with bulged ends, the outline resembling closely that of the bed bug. In this form it is common to observe darker staining dots at one or both ends. The bacillus is a moderately strict anaerobe. Growth in meat broth results in the meat turning pinkish in colour, without

any blackening or signs of digestion. The odour is sour owing to the production of butyric acid, and some gas may be formed. In milk, a clot is produced and gas evolved, but the alterations are never so striking as in the "stormy fermentation" of *Cl. welchii*. Gelatin is liquefied. Glucose, lactose, and maltose are fermented, but not saccharose. The organism is less actively saccharolytic than *Cl. welchii* and is not proteolytic. The bacillus is actively hæmolytic. The spores are highly resistant and can survive boiling for some time.

*Cl. septicum* is found in the soil and its natural habitat is probably in the intestines of animals, chiefly herbivora. Its virulence varies greatly with the strain, some types being almost or completely non-pathogenic. Its importance to man is that it is one of the chief organisms capable of producing gas gangrene, although it is much less commonly found in this condition than *Cl. welchii*. The form due to this organism differs somewhat from that due to the *Cl. welchii* in that oedema with blood-stained fluid is more marked, while necrosis and gas production are less evident. The bacillus is rarely found alone in such wounds, but pure infections have been recorded.

Subcutaneous inoculation of guinea-pigs, mice, or rabbits with a virulent strain produces similar lesions with death in from 24 to 48 hours. In the guinea-pig, a terminal septicæmia is usually observed, and one of the steps used in isolating the organism in pure culture is to inject the material containing the bacilli subcutaneously and to make cultures from the heart's blood after death. In the mouse, subcutaneous injection always produces an acute and fatal septicæmia.

A soluble toxin is found in cultures, after from 24 to 48 hours. Intravenous injection of 0.1 to 2.0 c.cs. into a rabbit causes death in from 3 to 15 minutes. With smaller doses, death may occur in from 1 to 12 hours. Its local actions are to produce oedema and necrosis and also to cause hæmolysis. An antitoxic serum (which may also be anti-bacterial) has been produced, and this is of great use prophylactically and is also of benefit therapeutically in the early stages of gas gangrene.

**Clostridium oedematiens.** (*B. novyi*, *B. oedematiens*), *Clostridium novyi*

Novy, 1894, Weinberg and Sequin, 1918.

This bacillus, which measures from 3 to  $10\mu$  by  $0.8$  to  $1.0\mu$ , resembles in appearance *Cl. welchii*, but is usually rather longer. It occurs singly, or in pairs, but long chains may be present in culture. Curved (C) forms are frequently seen, and a pair of such bacilli, curved in opposite directions, produces an S form. It has no capsule and, although it is supplied with flagella, it is never actively motile. Spores, which are oval, thicker than the bacilli and subterminal, are freely produced in culture, even in the presence of fermentable carbohydrate. In young cultures the bacilli are Gram positive, but later fail to retain the stain.

It is one of the strictest of the anaerobes and care is required for its cultivation. In glucose broth a uniform turbidity is first produced, but in a short time the bacilli fall to the bottom of the tube, and later the culture becomes clear owing to rapid autolysis. Glucose and maltose are fermented, with the production of acid and gas, but not lactose or saccharose. Meat broth is not darkened, but the meat may acquire a slightly pinkish colour and a few bubbles of gas may be evolved. Gelatin is slowly liquefied. The spores may remain viable after 30 minutes' exposure to  $100^{\circ}$  C. in the wet state.

*Cl. oedematiens* is probably second in importance only to *Cl. welchii* in the causation of gas gangrene. The form of disease produced differs considerably from that due to *Cl. welchii*, since the local condition is chiefly one of intense oedema; necrosis and gas formation are not marked features of its action. The bacilli have occasionally been found in the blood stream, but septicæmia is only a terminal phenomenon. The chief features of infection are due to the intense general toxæmia which results from its invasion of the body. It is pathogenic for the usual laboratory animals, injection of cultures leading to a widespread gelatinous oedema, intoxication and death. A soluble toxin is produced in the first few

days of growth and filtrates may have a small lethal dose, 0.05 c.c. being frequently sufficient to kill a guinea-pig when injected subcutaneously. The action of the toxin locally is similar to that of the living bacilli (the production of a gelatinous oedema) and the interval between the injection and the death of the animal may lie between 6 and 48 hours, depending on the size of the dose. Filtrates are also hæmolytic but not strongly so.

Antitoxic sera have been prepared by the inoculation of horses. These have great protective powers in experimental animals, and have been used, often with considerable success, in man in the treatment of gas gangrene due to this organism.

#### METHOD USED FOR THE RAPID IDENTIFICATION OF THE CAUSATIVE ORGANISM OF GAS GANGRENE

Practically all cases of gas gangrene in the European War were due to one or more of three organisms—*Cl. welchii*, *Cl. septicum*, and *Cl. oedematiens*. In the later stages of the war efficient antisera were prepared against these, and the need arose for some more rapid means of identification than that supplied by the usual cultural methods.

The method devised was based on the protective power of the antisera. An emulsion of the necrotic tissue from the wound was prepared and divided into five parts. The first was untreated. To the second was added 1.0 c.c. of anti-*Cl. welchii* serum, to the third 1.0 c.c. of anti-*Cl. septicum* serum, to the fourth a similar amount of anti-*Cl. oedematiens* serum. 1.0 c.c. of each serum was added to the fifth tube. Five guinea-pigs were inoculated with the five batches and the results were usually readable inside twenty-four hours. If *Cl. welchii* alone was present, the first, third and fourth animals sickened and died; the second and fifth were unaffected, being protected by the anti-*Cl. welchii* serum. Similarly single infections with either of the other organisms could be diagnosed. Where a double or triple infection existed only the fifth animal escaped, but in the case of a double infection the one of the

three guinea-pigs (second, third or fourth) which was totally unprotected succumbed more quickly than the other two, each of which was protected against a single bacillus.

***Clostridium histolyticum.* (*B. histolyticus*).**

Weinberg and Sequin, 1918.

*Cl. histolyticum* measures from 2 to  $6\mu$  by  $0.8\mu$ . It is most commonly found as a straight or slightly curved, non-capsulated bacillus in pairs or, less frequently, in short chains. It is actively motile. Spores are readily produced in cultures and are oval and terminal or sub-terminal in position. Young bacilli are Gram positive, older forms failing to retain the stain.

*Cl. histolyticum* grows fairly readily under anaerobic conditions and is actively proteolytic. Meat is blackened and digested and, in the deposit remaining, bundles of white crystals (tyrosin) are frequently found. In milk a fine clot is produced, and subsequently digestion occurs. Gelatin and coagulated serum are liquefied. No carbohydrate is fermented, with the possible exceptions of glucose and maltose, on which the organism may have a slight and slow action. All cultures have a foul and nauseating odour.

The organism is seldom if ever found alone as a cause of disease in man, but it occurs fairly frequently in contaminated "war wounds." In these its action is chiefly to produce necrosis and softening of the muscles. When injected into a guinea-pig, it causes widespread necrosis and gangrene. By its growth a soluble toxin is produced which reaches the maximum potency within one or two days. Intravenous inoculation of a guinea-pig with the toxin may cause the death of the animal in a few minutes.

***Clostridium sporogenes.* (*B. sporogenes*).**

Metchnikoff, 1908.

*Cl. sporogenes*, which measures from 2.5 to  $7\mu$  by  $0.6$  to  $1.0\mu$ , is a motile organism which does not form capsules. It is

found usually singly or in pairs, but some short chains may appear in cultures. It is Gram positive but, like many of the anaerobic bacilli, older organisms may be Gram negative. Oval spores, which are sub-terminal, are produced freely in cultures, even in the presence of carbohydrates, but are less common in wounds.

The bacillus grows readily under anaerobic conditions, and is capable of growth in the presence of an amount of oxygen sufficient to inhibit the development of many of the other anaerobic bacteria. In meat broth it grows rapidly and the meat is darkened or even blackened, especially if a piece of iron be present, and subsequently almost completely digested. Some gas is evolved, and the culture has a foul, putrefying odour. In milk there is some coagulation with later digestion. Gelatin, coagulated serum, and egg white are all liquefied. The organism is actively proteolytic, but its saccharolytic powers are restricted, glucose and maltose being the chief carbohydrates fermented.

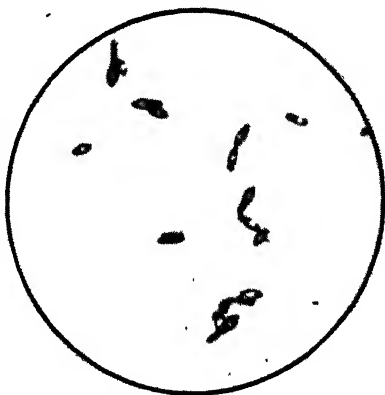


FIG. 78.—*CL. SPOROGENES* FROM BROTH CULTURE ( $\times 950$ ).

*Cl. sporogenes* is found in the faeces of man and animals, in the soil and in water. It is one of the commonest contaminants found in laboratory media intended for anaerobic culture, and many "pure cultures" of anaerobic bacilli contain it, in addition to the organism known to be present. Its powers of resistance are so high as to render it difficult to free media from it, since the spores may resist boiling for one hour and even a temperature of  $115^{\circ}$  C. in the autoclave for the same time.

It is one of the commonest organisms found in "gas



gangrene" wounds and, although alone it is almost or completely non-pathogenic, it is of importance in that its presence appears to be able to exalt the virulence of the pathogenic bacteria, particularly of *Cl. welchii*. In addition to this it is able, thanks to its proteolytic properties, to digest dead tissue, but it appears to be without action on living tissues

## CHAPTER XLIII

### ACTINOMYCOSIS

#### **Actinomyces bovis.**

Harz, 1877.

THE name Actinomycosis is given to a disease of animals (chiefly cattle) and man which is characterized by the formation of granulation tissue accompanied by suppuration. The chief characteristic of the process is the occurrence in the lesions of granules or colonies of the causative organism, the *Actinomyces bovis* or ray fungus. These granules, which are frequently yellowish (sulphur granules) or greyish in colour, may be easily visible to the naked eye or may be of microscopic dimensions. When examined unstained under the microscope, it is possible to make out something of their structure. The central portion is composed of a dense felted mass of filaments which show true branching. Each filament has a diameter of about  $0.5\mu$ , and appears to be composed of a rather granular protoplasm surrounded by a delicate sheath. In older colonies the filaments fragment, and may resemble cocci. These fragments were formerly supposed to be spores, but this is almost certainly incorrect. Radiating from the central mass are filaments which, as they approach the free surface, terminate in swollen, homogeneous, pear-shaped bodies, the so-called clubs. These clubs, which are usually regarded as very characteristic of actinomyces, are not reproductive organs, but are supposed to represent either a degenerative process or a means of defence of the parasite against its host's leucocytes. The fact that similar clubs are found in the bodies of animals inoculated with the *Actinobacillus*, various acid-fast bacilli and even inorganic

substances, strongly suggests that the clubs have origin in the host rather than in the parasite, but histologists do not appear able to explain their true nature. When stained, the filaments are found to be Gram positive, but the clubs do not retain the stain. In young colonies the clubs may be exceedingly delicate and be dissolved in water, but in older colonies they can be demonstrated either unstained or by the counter-stain in Gram's method. A better method of demonstrating clubs in sections is to stain with hæmatoxylin for 2 minutes, wash, stain with strong carbol-fuchsin for 2 minutes, wash and decolorize with alcoholic picric acid for 1 minute.

The first recorded culture was made by Boström.



FIG. 79.—*ACTINOMYCES BOVIS* ( $\times 200$ ).

This was of an aerobic organism which, although it is probably responsible for some cases of actinomycosis, is much less common than the organism described by Israel and Wolff. This is a partial anaerobe; that is, it grows best in the presence of a small amount of air. Cultures are made by mixing a washed and crushed granule from an uncon-

taminated lesion with melted glucose agar, cooled to about  $50^{\circ}$  C. After mixing, the inoculated medium is allowed to cool in tubes. After a few days' incubation at  $37^{\circ}$  C. the colonies are visible as whitish, irregular specks which are most numerous and best developed in a zone about 0.5 to 1.5 cms. from the surface of the medium. The colonies, which may ultimately grow to a diameter of 2 to 3 mms., are composed of radially arranged, branching filaments. In glucose broth irregular masses occur at the bottom of the tube. In these media clubs are not produced, but if serum be added

there is some swelling of the ends of the filaments. Another satisfactory medium is glucose broth containing a few drops of fresh blood. This may be covered with a thin layer of oil. Colonies like puff-balls form at the bottom of the tube. The organism grows best at body temperature and but little, if at all, at 20° C.

In cattle the parasite affects chiefly the tongue (woody tongue) and jaws, producing lesions in which the predominant feature is granulation tissue rather than suppuration. The nodules are composed chiefly of polymorphonuclear leucocytes, round cells, both small and large, and plasma cells. There is a considerable proliferation of the surrounding connective tissue, and later the centre of the mass may undergo softening, leading to the appearance of sinuses. The disease is very chronic and may become healed with subsequent calcification, or may cause death by direct spread. Metastatic spread is rare and constitutional disturbances are slight, pointing to the absence of any considerable degree of toxin production.

In man the parts affected

are commonly about the mouth, but the skin of any part of the body and the digestive tract, the liver, and lungs are also attacked. A striking feature of the disease is the absence of involvement of lymph glands. The lesions are more suppurative than in cattle and chronic abscesses with discharging sinuses are produced. Death is not commonly caused, except by direct spread to vital organs or by the addition of another infection.

The method of spread of the disease is still in doubt, but is most probably from case to case, although it is frequently

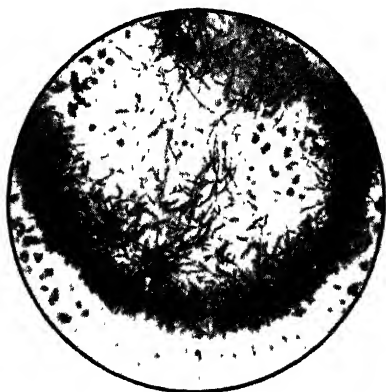


FIG. 80.—COLONY OF ACTINOMYCES BOVIS IN LIVER ( $\times 500$ ).

impossible to establish this. From the finding of grain, chiefly barley, in actinomycotic lesions it has been supposed that the parasite is introduced with the grain, and this may be true of Boström's organism, but almost certainly not of the *Actinomyces* of Israel and Wolff. Some believe that the organism is a common mouth saprophyte which may, for some unknown reason, become pathogenic.

Inoculation of animals (guinea-pigs and rabbits), with granules from the body or with cultures, has given rise to nodules similar to those occurring in the natural disease. In



FIG. 81.—STREPTOTHRIX IN PUS  
( $\times 950$ ).

these nodules the typical actinomycotic granules are to be found. In addition to the *Actinomyces*, a pleomorphic Gram-negative bacillus (*Actinobacillus actinomycetem-comitans*) is quite commonly found in actinomycotic lesions. Its relation to *Actinomyces*, and to the disease is, as yet, uncertain.

In tropical countries a chronic type of inflammation, with suppuration and sinus formation, usually in the neighbourhood of the foot (Madura foot), is of fairly common occurrence. In the majority of the cases, in which yellow granules are found, the causative organism is *Actinomyces maduræ*, which resembles *Actinomyces bovis*. No clubs are present in the granules. This organism is a strict aerobe. From the lesions of many cattle suffering from a disease resembling actinomycosis, with the occurrence of granules supplied with clubs, a small Gram negative, aerobic bacillus has been isolated by Lignières and Spitz. Pure cultures of this bacillus produced, in guinea-pigs and oxen inoculated with it, lesions similar to those of true actinomycosis in which typical granules with clubs were

present. The name *Actinobacillus lignieresii* has been given to this organism. Very few human cases have been recorded.

In man, chronic suppurative conditions are occasionally due to an organism showing a branched mycelium without granule formation. Such streptothrix infections we distinguish from actinomycosis, which term ought, we believe, to be confined to lesions in which definite granules are produced.

## CHAPTER XLIV

### DISEASES DUE TO VIRUSES AND TO RICKETTSIÆ

IN the majority of the diseases which we have already considered the causative organism can be seen with the aid of the microscope and can be cultivated on artificial media. There remain a number of diseases affecting men, animals and plants in which it is usually impossible either to see or to cultivate the responsible agents. It may be that the methods we have used are unsuitable—it was a considerable time before the *Tr. pallidum* was discovered and then several years before it was cultivated, if, indeed, it ever has been cultivated—but we have reason to believe that at least some of the active agents of these diseases are too small to be seen with any microscope. Optical theory proves that the limit of resolution obtainable with the best microscope is about  $0.25\mu$ . This means that, although a smaller object may be rendered visible by special illumination, its shape cannot be made out; it appears merely as a dot. For this reason these agents are sometimes called ultra-microscopic viruses. This term is unsatisfactory as modern improvements in staining methods have resulted in the development of stains which exaggerate the size of these organisms, so that some of them may easily be seen in stained preparations.

By filtration we have another means of gauging the size of these agents. Bacterial filters may be made of various degrees of fineness. By the use of a series of graded filters we could select those which would hold back the anthrax bacillus, *Bact. coli*, and the influenza bacillus respectively. A still finer filter would prevent the passage of all the known bacteria, but the filtrate might contain an agent capable of producing

such a disease as measles. Such agents are often called filterable viruses. Neither ultra-microscopic nor filterable are satisfactory terms, and we propose to call these organisms merely viruses. We cannot easily define a virus, but the connotation of the term will become clearer when we learn something more about them.

Since many of the viruses are invisible and cannot be cultivated on artificial medium and can, in fact, only be recognized by the effects which they produce, they are regarded by some as more an object of philosophic theory than of scientific examination, and so we have various schools of thought which attempt to show that they differ in almost all respects from bacteria. For some they are of the nature of enzymes, and for others in a condition midway between living and non-living matter. The fact that certain viruses of plant diseases have been obtained in the form of protein crystals or fibres has lent support to those who believe that viruses are non-living. But there is no certainty that plant and animal viruses are identical and a crystalline form of life is not inherently impossible. Until good reasons are given for believing otherwise, we propose to consider the viruses as living organisms essentially similar to bacteria.

Since filtration is an important process in the study of viruses, the theory of filtration deserves consideration. Very little observation teaches us that a filter is much more than a sieve which grades the size of particles into those small enough to pass through and those so large that they are held back. Through the walls of the filter run tortuous channels. Unless the particles in the fluid we are filtering are smaller than the diameter of these, they cannot pass through except under the influence of great pressure which might distort them sufficiently to force them through. But some bodies which are definitely smaller than the pores also fail to pass. This is because they adhere to the particles of which the filter is composed in virtue of a mutual electrical attraction, just as steel filings would not pass through a magnetized sieve, even though they could freely pass through a copper sieve of the same mesh. Most of our bacterial filters, such as the



Chamberland and Berkefeld, are composed mainly of silicates and carry a negative charge. Positively charged particles will therefore adhere to or be adsorbed on the material of which the filter is composed, irrespective of their size. An experiment with two filters, one Berkefeld (negative charge) and the other made of plaster of Paris (positive charge), and solutions of two dyes, Victoria blue (positive charge) and Congo red (negative charge), will illustrate the importance of charge. If we attempt to filter the two solutions through the Berkefeld filter we find that the Congo red dye passes through but that the filtrate from the Victoria blue solution is colourless. The positively charged molecules of the latter have been adsorbed on the filter. With the plaster of Paris filter the reverse occurs—the Victoria blue solution passes through unchanged, but the Congo red molecules are held back. If to the solution of Congo red we add a small amount of acid, which changes the red colour to blue and also alters the charge on the molecules from negative to positive, we find that the dye behaves differently. It now passes through the plaster of Paris filter but is held back by the Berkefeld. With both the dyes the molecules are very much smaller than the pores, and whether they pass or fail to pass through a filter depends on their charge and on the charge of the materials composing the filter. The question of the filterability of viruses is intimately associated with the charge they carry, which may depend on the reaction of the fluid in which they are suspended, and also on the type of filter. It follows that, in any description of a filtration experiment, these factors should be stated. Even very minute bodies may fail to pass a filter, not because they are adsorbed on the filter but because they have already been adsorbed on relatively large protein particles, incapable of passing through the pores. Inability to pass a filter does not, therefore, prove that a virus is, in itself, too large to do so.

High pressures should be avoided in filtration owing to the danger of forcing through the pores relatively large organisms by distortion. On the other hand, the pressure must be sufficiently high to prevent the operation of filtration occupying too long a time for, if the process is very slow, some long,

fine organisms (e.g. spirochaetes) which would not normally pass through, may grow through the pores and appear in the filtrate.

Collodion filters, which act purely as sieves and avoid the fallacies due to adsorption phenomena, can be made of varying degrees of fineness and, by their use, it has been found possible to estimate the size of the viruses, some of which are as small as  $8\text{ m}\mu$  and others as large as  $175\text{ m}\mu$ . The millimicron ( $\text{m}\mu$ ), which is the unit of measurement employed for these small bodies, is equal to  $\frac{1}{1000}\mu$ .

Their small size is the first characteristic of viruses which we have discussed. We must now turn to the question of their cultivation. Only one has been unquestionably cultivated on ordinary media, that of pleuro-pneumonia of cattle, and concerning it there is doubt whether it is to be

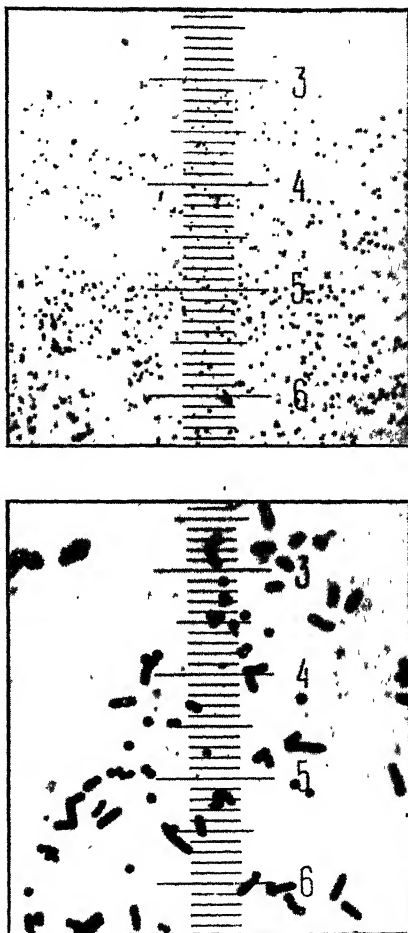


FIG 82.—VIRUSES AND BACTERIA.

A—Elementary Bodies of Vaccinia Virus from conjunctiva of rabbit; B—Staphylococci and Bact. coli. Both A and B stained by Paschen's Stain. Both  $\times 1200$ . Each division on micrometer scale =  $1.1\mu$ .

regarded as a bacterium or a virus. It is just on the limit of microscopic visibility, and it is convenient to regard it as being a link connecting the bacteria and the viruses. Several viruses have definitely been cultivated outside the body, but only in the presence of cells, and usually only when these cells are themselves living and growing as in a tissue culture. The failure to culture viruses may be due to imperfections in technique, which may be overcome, or it may be because the viruses are obligatory cell parasites which can only grow inside cells.

A plausible theory is that viruses are obligatory cell parasites because they are devoid of the power of producing enzymes. For this reason, they are unable to alter food material and to make it suitable for absorption, as are the bacteria: they must be supplied with food ready for utilization by them, and it is only within cells that this is available.

Cultures of viruses may be obtained in cultures of tissue cells which they can invade. A tissue culture of minced chick embryo in Tyrode solution is suitable for some. A simple method of obtaining cultures of many of the viruses—those of vaccinia, psittacosis, and influenza for example—is to inoculate material containing the virus on to the chorio-allantoic membrane of a chick embryo developing within its shell. A hen egg, incubated for 10 days at 41° C., is a suitable medium. A hole is bored in the shell, with aseptic precautions, and the material is injected from a syringe, the needle of which has penetrated the shell membrane and is in contact with the chorio-allantoic membrane. After the hole is sealed, the egg is incubated for a further 3 or 4 days, by which time the virus has multiplied and produced extensive lesions in the membrane.

The various viruses differ as much one from another as do bacteria in respect of the effects of physical and chemical agents. Some are destroyed by a temperature of from 45° to 55° C., others can resist 70° C. for a time; some die rapidly when dried, others may survive in the dry state for years; some are most easily killed by one disinfectant, others by a different one. Most of them show one point of difference from

the majority of bacteria—they can survive for long periods in 50 per cent. glycerol, which is fatal to practically all non-sporing bacteria.

The degree of infectivity of the diseases produced by viruses is quite varied. In some actual contact with an abraded surface is necessary in order to infect (rabies), while in others the virus can travel for a short distance through the air and, apparently, enter the body through an uninjured mucous membrane (small-pox).

Certain viruses act solely or mainly on a particular tissue: this is called tropism. A virus may be dermatropic (affecting ectodermal cells), neurotropic (affecting nerve cells), viscerotropic (affecting the cells of organs) or pantropic (affecting cells in various parts of the body). By suitable animal passage, the tropism of a virus may be altered: it may be changed from being dermatropic to become neurotropic. The majority of viruses causing disease in man are either neurotropic or dermatropic.

It is sometimes claimed that the immunity against virus diseases is much higher than that against bacterial diseases. There are considerable exceptions to such a generalization. It is true that a very solid immunity results from an attack of small-pox, but not more so than is the case with typhoid fever. The temporary nature of the immunity to the pneumococcus is equalled by the fleeting immunity following infection with the virus of herpes, the common cold and influenza.

The serum of a convalescent man or animal contains antiviral bodies. These may be shown by their neutralizing power, by flocculation or agglutination, and by complement fixation. The neutralizing power is demonstrated by injecting a mixture of virus and antiserum into a susceptible animal: disease does not result. For flocculation, agglutination, and complement fixation reactions, a suspension of elementary bodies is obtained by grinding affected tissues and centrifuging or filtering to remove cell debris.

After an attack of certain virus diseases, as after certain bacterial diseases, the virus may remain in the tissues, as may

the bacteria, without inflicting apparent damage. We may have healthy virus carriers, as we have healthy bacteria carriers.

In practically all these points we see the essential similarity between viruses and bacteria, rather than any striking dissimilarity. There is just one other characteristic in which the viruses do seem to be unique. This is the occurrence, during the diseases, of what are called "cell inclusions." These, in many cases, are so typical as to act as useful diagnostic agents, but between those occurring in different diseases there is little similarity. Some affect the nucleus and others

the cytoplasm. Since viruses are essentially intracellular in their habits, while bacteria are extracellular, it is not surprising that abnormalities in affected cells should appear. There is no general agreement as to the nature of cell inclusions, but the most probable theory is that they are aggregates of virus particles.



FIG. 83.—ELEMENTARY BODIES OF A VIRUS (ECTROMELIA), CAUSING DISEASE IN MICE, AS SEEN WITH DARK GROUND ILLUMINATION ( $\times 600$ ).

In preparations made from a tissue infected with a virus, very small coccoid bodies may, after

appropriate staining, be seen lying free from the cells. These are the elementary bodies and there is little doubt that these are particles of virus.

As will be seen from the above, our knowledge with regard to the causes of many of these diseases is very scanty. At present a large amount of experimental work is being carried out in connection with them, and it is probable that we shall be able, in a few years, to give a more complete review of the viruses than is now possible.

### Rabies.

Rabies or hydrophobia is a disease affecting practically all mammals, but occurring with greatest frequency in dogs and wolves. Infection is conveyed by the saliva of a rabid animal coming in contact with a cut or abrasion of the skin, most usually as the result of biting.

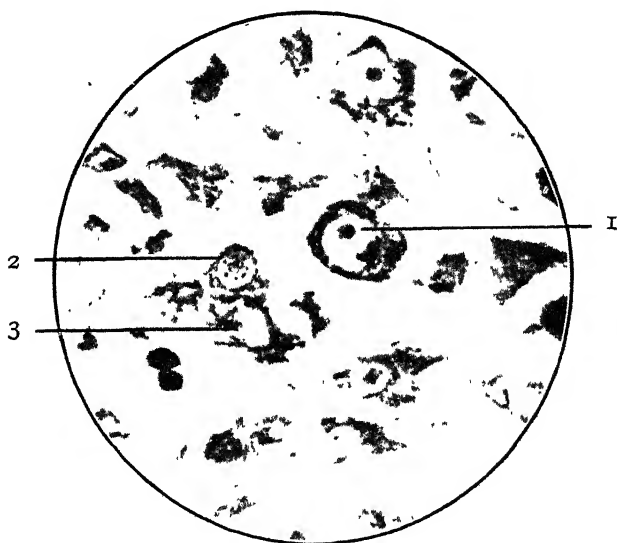


FIG. 84.—NEGRI BODY IN NERVE CELL ( $\times 850$ ).

1. Nucleus of nerve cell.
2. Degenerated nucleus of nerve cell.
3. Negri body showing a rosette-like arrangement of its granules around a central structure.

In man there may be, after an incubation period of from 15 days to 2 months (rarely up to 7 months), a prodromal pain in the region of the wound and considerable depression. Breathing becomes difficult and swallowing is painful, owing to convulsive contractions of the muscles of the throat. This is frequently a very outstanding symptom and the fear of bringing on a spasm may be so intense that even the thought

of swallowing anything, not merely water, may bring on an attack. It was this that gave rise to the belief that the victim feared water, and hence the name hydrophobia. Spasms become general in all parts of the body and all reflexes are increased. The temperature is elevated, the pulse rapid, and delirium is frequently present. Later, weakness comes on and, after a short paralytic stage, death occurs, usually after from three to seven days' illness.

The post-mortem evidence of the disease is not striking. There may be some congestion with minute hæmorrhages in the central nervous system and a leucocytic invasion of the perivascular lymphatic spaces. Degenerations of some nerve cells, particularly in the anterior cornua and in the cranial nerve centres, is of common occurrence. In certain nerve cells of the cortex, of the anterior cornua, of the hippocampus, and of the grey matter of the cerebellum and also lying free, bodies are seen which are characteristic of rabies, being almost invariably present, and which are not found in any other condition. These, the Negri bodies, may be seen either in smears or sections of brain tissue taken from the areas indicated. They vary greatly in size, from 0.5 to 18 $\mu$ , and may be round, oval, or irregular in shape. Their outline is sharp and definite and, in the interior of the larger bodies, may be seen from one to sixteen chromatin granules. Similar bodies have been noted in the salivary glands. As in the case of other inclusion bodies, the exact nature of the Negri bodies is doubtful, but most authorities believe that they are or contain aggregations of the viri of the disease.

The virus is capable of passing through a Berkefeld and the coarser Chamberland filters. Various claims to have cultivated it have been made but have not been substantiated.

Although we have so little certain knowledge of the nature of the organism of rabies, we know a considerable amount concerning its mode of action. The virus affects chiefly the central nervous system, but is also present in the salivary glands and so in the saliva. As has been proved in a number of cases, the saliva is infective

several days before the appearance of the first signs of the disease. In the natural disease in man infection is almost always from the bite of a dog, wolf, or other rabid or pre-rabid animal. Not all, however, bitten by such an animal and untreated acquire rabies, but only about 16 per cent. The incidence appears to depend chiefly on the severity and depth of the bite, its locality and the amount of saliva which comes in contact with the wound. Bites on the face and hands are most dangerous, while those through clothing are least. The influence of locality, apart from such accidental circumstances as the amount of clothing encountered, depends chiefly on the nerve supply of the part. A region richly endowed with nerves is the most favourable for the development of the disease, for the virus, like the toxin of tetanus, passes up the nerves to the cord and thence to the brain. It is not, however, as in the case of tetanus, merely a toxin which passes to the central nervous system but the living organism, for the brains of rabid animals on injection can reproduce the disease indefinitely in series. The virus is easily killed by heating at 50° C. for one hour, or at 60° C. in less than half an hour, by 5 per cent. phenol in one hour and by drying in a few days.

Animals may be infected most certainly by sub-dural inoculation through a trephine opening in the skull, of an emulsion of any part of the central nervous system, of the cerebro-spinal fluid or of the saliva of a rabid animal. Inoculation with such material into the anterior chamber of the eye or into a large nerve trunk is almost as effective. Rabies also results, in a high proportion of animals, from the intramuscular or subcutaneous injection of material containing virus. Injections of blood or of emulsions of the internal organs do not reproduce the disease.

Pasteur discovered that the virulence of the virus could be modified by passage through a series of animals. The virus found in the nervous system of the naturally infected dog of the Paris streets, usually known as "Street virus," had an incubation period when injected subdurally in rabbits of about 12 to 14 days. After passage through a series of rabbits



its incubation period was reduced and it caused paresis on the sixth or seventh day in rabbits, but it had then lost most of its pathogenicity for the dog and also for man. Further passage did not increase its virulence for rabbits, and so it was known as "*Virus fixe*." This procedure enabled any one to obtain a virus of practically constant virulence. Pasteur found that by drying the spinal cord of a rabbit infected with *virus fixe* he apparently could reduce still further the virulence of its contained virus. A spinal cord dried for 6 to 8 days was of very slight virulence, while 14 days of drying completely deprived it of all its pathogenicity. As a result of his animal experiments, Pasteur was led to try to produce an artificial immunity in a boy bitten by a rabid dog. Hope was given by the fact that immunity could be produced rapidly in animals, while in man the natural disease had a long incubation period. If the immunizing process was completed before the elapse of the period of incubation, the disease would not appear. Pasteur's first test was successful, and many years of brilliant successes with many thousands of lives saved have rendered testimony to the essential correctness of Pasteur's theories. The process cannot be regarded as therapeutic, but rather prophylactic; active immunity must be produced before the disease declares itself, for once rabies appears death is certain to follow. Without treatment about 16 per cent. of those bitten by proved rabid animals die; with Pasteur's treatment the mortality is less than 1 per cent., probably about 0.5 per cent.

In the original Pasteur method the patient received, daily or twice daily, a subcutaneous inoculation of a suspension, in saline, of about 1.0 cm. of a cord taken from a rabbit dying with *virus fixe* infection. For earlier injections, cords which had been dried over caustic potash for 14 days were used and, in succeeding injections, cords dried for shorter times until for the last injection, from 14 to 21 days after the commencement of the course, a cord dried for only one day was employed. Pasteur believed that the drying process reduced the virulence of the virus. Actually it appears to cause the death of virus, very little, if any, remaining alive

after 10 days' drying. Since 1911 the Pasteur Institute has employed, for injections, cords dried for varying times and suspended in glycerol, in which the vitality of the virus is preserved, until required.

Semple has simplified the technique and has eliminated the necessity for the patient to go to a Pasteur Institute for treatment by using virus which has been killed by emulsifying the cords in phenol solution. The dose is gradually increased at each injection and, since the organisms are dead, the vaccine keeps well and can be used anywhere. His results appear to be equally as good as those of workers who follow Pasteur's system as originally devised. There is no doubt that effective immunity can be produced by dead rabies virus.

The serum of animals immunized against rabies has been used therapeutically without result. It is, however, given prophylactically in conjunction with vaccine in some institutes, and appears to be definitely virucidal.

As regards the prophylaxis of rabies the most important step is to abolish rabies in dogs. That this can be done by quarantining all imported dogs and enforcing muzzling, when the disease breaks out, is shown by the great success of these measures in keeping Great Britain and Ireland free of the disease for many years. Thorough cauterization of bites, either with the actual cautery or with fuming nitric acid, if applied within a short time, although even at the end of several hours it is not without effect, very greatly reduces the risk of contracting the disease. All those bitten by proved rabid animals should be treated with anti-rabies vaccine, either at a Pasteur Institute or by Semple's method.

Diagnosis of the disease in dogs is of great importance. It is made by the finding of Negri bodies in the brain, or by the subdural inoculation of rabbits, guinea-pigs or mice with an emulsion of the brain of the animal. The only serious disadvantage of this, the most certain method, is the time which must elapse (14 to 21 days) before the inoculated animal develops typical symptoms.

### **Epidemic Poliomyelitis.**

This is a disease of childhood, but adults are not entirely immune. It occurs chiefly in sporadic form, but from time to time epidemics, which may assume considerable proportions, occur. The onset of the disease is usually abrupt with pyrexia, frequently accompanied by sore throat. In a few days weakness of a group of muscles, usually in a limb, is observed and this may advance to paralysis. The mortality is low but, as a result of the disease, a considerable degree of deformity is produced, owing to paralysis, although the extent of this, after recovery from the disease, is not so great as in the acute stage.

The pathological change, which may be present in any part of the central nervous system but is of most frequent occurrence in the cervical and lumbar regions of the cord, is chiefly an acute inflammation with hyperæmia and an exudation of mononuclear leucocytes into the perivascular lymphatic spaces. Thrombosis or rupture of small vessels is commonly present. The changes are most marked in the anterior commissure and in the anterior horns of grey matter. As a result, some of the nerve cells die with subsequent degeneration of the motor fibres. The meninges, posterior ganglia and posterior cornua, while similarly affected during the acute stage, undergo little permanent damage. In the cerebro-spinal fluid, a moderate number of polymorphonuclear leucocytes may be found at the onset, being later replaced by mononuclear cells.

The virus, which is very minute (8 to 12 $\mu$ ), is capable of passing through Chamberland and Berkefeld filters. It is readily killed by heating, a temperature of 45° to 55° C. destroying it in less than half an hour, but it resists drying and cold well, and may survive for more than a month at -2° C. It is easily killed by potassium permanganate and hydrogen peroxide. The virus is present, in greatest concentration, in the central nervous system, but has also been found in lymphatic glands, the salivary glands, the mucous membrane of the naso-pharynx, and in saliva and nasal secretions. It

is occasionally present in urine and fæces. It is not usually found in the cerebro-spinal fluid, and appears to be absent from the blood and solid organs.

The disease is transmissible to both the higher apes and lower monkeys, and also to rabbits. Infection may be produced in monkeys by injection of emulsions of the brain, cord or other affected parts of the body of an individual suffering from the disease. The disease, which clinically and pathologically resembles that of man, is most certainly produced by subdural or intracerebral inoculation (employed to supply the only certain method of diagnosis of the disease post-mortem), but other routes, intraperitoneal, intrathecal, subcutaneous and into a large nerve have also been successful. Intravenous inoculation but rarely produces the disease. Of great interest, epidemiologically, is the fact that monkeys have acquired poliomyelitis as a result of applying material, containing the virus, to the mucous membrane of the nose, previously scarified, or even by the inhalation of such material. In monkeys the incubation period is, most commonly, from eight to nine days, but may be over a month.

Poliomyelitis is spread from a case or from a convalescent or healthy carrier to the victim, either directly or indirectly, and almost certainly the chief transmitting agents are the saliva and nasal secretions. The virus has been found in the nasal mucous membrane for some months subsequent to the disease, and also in that of a monkey several months after intracerebral inoculation. It probably passes from the nose to the brain and cord by way of the olfactory nerves. One attack of the disease, in almost all cases, appears to give complete immunity and an artificially infected monkey, after recovery, is insusceptible to further infection. The serum of a recovered case or of an immunized monkey is capable of neutralizing the virus, but it is very doubtful if such serum has ever been successful in the treatment of the disease. Convalescent serum or, if that be unobtainable, adult serum may be used to confer passive immunity. There are some grounds for believing that 30 c.cs. of such serum injected intramuscularly may be useful as a prophylactic

measure. Attempts have been made to secure active immunity by the use of virus. Killed virus appears to have no immunizing power and living virus, even after its virulence has been reduced, has proved dangerous.

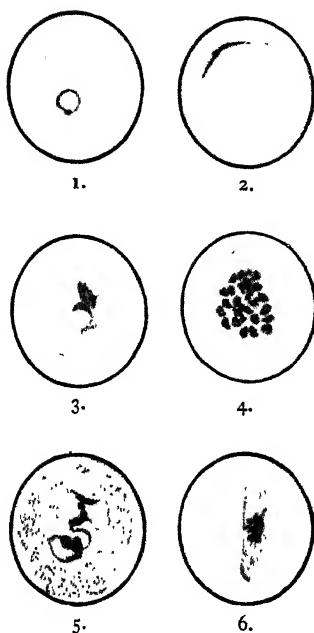
### **Encephalitis Lethargica.**

This disease was first accurately described and differentiated from acute poliomyelitis, which, in many respects, it resembles, in 1917. Its clinical manifestations vary considerably both in character and severity. General signs of infection are usually slight, though constantly found at some time during the disease. The focal signs seem to depend on the region of the brain which suffers most from the infection. So we may find, to mention but a few, ocular palsies, mental symptoms or disturbances of muscular control, according as the oculomotor nuclei, cortex or basal ganglia are damaged. The course of the disease may be prolonged for many weeks and the fatality rate may be as high as 50 per cent., but probably would be lower if accurate diagnosis of all mild and abortive cases were possible.

The pathological changes observed are most marked in the grey matter in the upper part of the pons and in the region of the basal nuclei, particularly in and about the nucleus of the third cranial nerve. They are usually slight or absent in the other parts of the brain and the cord. Congestion of vessels and microscopic hæmorrhages may be observed, but the most constant finding is a perivascular infiltration ("cuffing") with large and small mononuclear cells, polymorphonuclear leucocytes being very rarely found. The alteration in the nerve cells is less marked than in poliomyelitis, but slight chromatolysis or even complete disappearance of Nissl granules has been recorded.

Da Fano and other workers have described the finding of "Minute bodies," very small coccus-like forms, irregularly round or oval in shape, usually single but occasionally in pairs. These bodies, which are present either within or without the nerve cells, may be the causative organism of the disease. Successful cultivations of the virus have been

# PLATE V.



## Malaria Parasites in blood films.

- |  |   |
|--|---|
| 1. <i>P. vivax</i> —Young ring form.                 | 4. <i>P. falciparum</i> —mature schizont.                             |
| 2. <i>P. falciparum</i> —Appliqué form on corpuscle. | 5. <i>P. vivax</i> —Amoeboid form :<br>Schuffner's dots in corpuscle. |
| 3. <i>P. vivax</i> —Amoeboid form.                   | 6. <i>P. falciparum</i> —Female Crescent<br>(Macrogametocyte).        |



claimed by certain workers but these are not generally accepted.

Investigation of a virus which cannot be demonstrated with certainty, either microscopically or culturally, must depend chiefly on animal experimentation, but the results obtained in this way with this virus are very inconclusive. In some cases, inoculation of rabbits has produced lesions resembling, in general, those occurring in the human disease but, in the majority, the results have been negative. The positive results are to be discounted for two reasons—the occurrence of spontaneous encephalitis in rabbits, and the similarity of the lesions produced to those caused by the virus of herpes, which may be present in various parts of the body of a person who has suffered from herpes. In the present state of our knowledge we can only say that it is doubtful if this virus has any pathogenicity for the lower animals.

The method of transmission of the disease is probably by way of the mucous membrane of the nose or naso-pharynx. Since contacts rarely develop the disease, it follows that the organism is but slightly invasive. The spread of encephalitis lethargica is, as seems probable on epidemiological grounds, chiefly due to the existence of healthy carriers in whom the virus is present in the nasal secretions, but who do not themselves acquire the disease.

### **Herpes.**

A virus is present in the vesicle fluid of Herpes Febrilis. When the cornea of a rabbit is inoculated with it a keratitis is produced and from this the disease may be transferred again to man. In the rabbit also, the virus, administered subdurally, is capable of producing a meningo-encephalitis which presents a pathological picture somewhat similar to that found in human encephalitis. The same condition may result from corneal inoculation with a highly virulent strain. It appears probable that the virus is widely distributed throughout the body in man: inoculation of rabbits with the cerebro-spinal fluid of a human case has produced typical lesions in the rabbits.



The virus can be cultivated in a tissue culture of the cells of rabbit testis in rabbit serum. The cultured virus gives rise to the same lesions in the rabbit as does human herpetic material and can reproduce the disease in man.

Little immunity is developed against the virus, and the same individual may suffer from repeated attacks of herpes. It is probable that the virus remains in the body between the attacks. The virus of Herpes Zoster is quite distinct from that of Herpes Febrilis. It is believed to be present fairly commonly in the central nervous system in man in a dormant condition, and to be activated by some injury to the nerve which becomes affected. On clinical grounds there appears to be some connection between zoster and varicella, and the viruses, both of which are relatively large (100 to 150m $\mu$ ), are serologically related.

### **Small-pox (Variola) and Cow-pox (Vaccinia).**

The viruses of small-pox and cow-pox are almost certainly the minute, but microscopically visible (125-175m $\mu$ ), Paschen bodies which are found at first free and later within the inflammatory cells of affected areas. The viruses are very resistant to glycerol, but are destroyed by a 1/10,000 dilution of potassium permanganate which, in such strengths, is practically inactive against the majority of bacteria. The viruses have been cultivated in tissue culture and on the chorio-allantoic membrane. A suspension of material from the papules produced, which contain Paschen bodies, has been used successfully for human vaccination.

Small-pox is an exceedingly infectious disease, but we are not aware of the route of infection. It seems probable, however, that the virus passes from the mucous membrane of some part of the upper respiratory tract of the patient to the same situation in the victim.

The relation between small-pox and cow-pox is of considerable interest. Material taken from a small-pox pustule does not, on inoculation in the calf, cause disease in every case, but in some there is a slight local inflammation and some signs of general sickness. By a few passages from calf to calf of

the material from the local lesions a virus is obtained which, in a calf, invariably produces local pustulation indistinguishable from true cow-pox. On transferring this to man, vaccinia and not variola results, and no matter how many passages are made from man to man, as was done when arm-to-arm vaccination was practised, variola does not reappear. There can be little doubt that the organisms of small-pox and cow-pox were originally the same, but that, by passage through calves, modification was effected so that the virus produces cow-pox and not small-pox. One of the strongest points in favour of the theory of identity is that small-pox protects against cow-pox and vice versa.

Many other animal poxes (sheep, swine, horse) are known. Their viruses are closely related to one another, as they are also to those of variola and vaccinia.

The diseases are of special interest as furnishing the first method of producing artificial immunity. Jenner observed that milkmaids, who had suffered from cow-pox as a result of milking infected cows, were immune to small-pox. He inoculated a boy with cow-pox material and later attempted to inoculate him with small-pox, but without effect. As a result of this and further experiments, he became convinced that an attack of one disease conferred a true immunity against the other. Vaccination has been widely practised and is, by every competent observer, acknowledged to be one of the greatest advances in medical science. The immunity to small-pox conferred by vaccination is not complete for life, and re-vaccination should be performed about puberty and probably ten years later. Those vaccinated within a period of five years or so are exceedingly unlikely to acquire small-pox, and even a single vaccination in infancy appears to furnish some protection throughout life as, in such persons, if small-pox does develop, it is of mild type and rarely fatal. Formerly vaccination was frequently done with material taken from the pustule of another child. To this arm-to-arm vaccination there were serious objections, and now "calf lymph" is used. This is prepared by wide vaccination on the abdominal skin of a healthy calf, very stringent precautions as to cleanliness

being taken. After five or six days the affected area is thoroughly washed, any crusts are removed and the vesicles are curetted off. The pulpy mass so obtained is ground in 50 to 80 per cent. glycerol solution and stored in the cold for some weeks. This serves to kill the majority of contaminating bacteria present, and does not injure the virus. When the lymph has passed bacteriological tests it is issued for use. If the method of producing virus in eggs develops satisfactorily it will make the supplying of virus for vaccination very much simpler and will have the great advantage of producing virus free from bacteria. The exact mechanism of the immunity is unknown, but it is certain that antibodies (virucidal, agglutinating, and complement fixing) appear in the serum. A mixture of lymph and the serum of a previously vaccinated calf is incapable of causing cow-pox in a normal animal. Although the lesion in vaccinia is purely local, the virus is probably widely disseminated through the body.

Occasionally, ten to fourteen days after vaccination, a condition of encephalitis or encephalo-myelitis develops. The cause of this post-vaccinal encephalitis is unknown, the chief theories being that it is due to vaccinia virus; that it is due to another virus contaminating the lymph used; or that it is the result of a lighting up of a virus previously present in the central nervous system in a dormant state. The administration of 5 c.cs. of the serum of a recently vaccinated person intrathecally or 10 c.cs. intravenously has been found beneficial, and the similar use of serum of an animal, immunized against vaccinia virus, has also given good results.

By the very rapid centrifuging of suspensions of tissues or tissue cultures containing Paschen bodies a practically pure suspension of these may be obtained. This may be used for the diagnosis of small-pox since the serum of the patient contains antibodies to them, the presence of which may be demonstrated by agglutination and complement fixation tests.

### **Yellow Fever.**

Yellow fever is a very fatal disease formerly common in endemic and epidemic form in tropical and subtropical

parts of America and Africa. The outstanding features of the disease are high temperature, great prostration, vomiting, hæmorrhages and jaundice. Infection is conveyed from man to man by mosquitoes, particularly *Aedes ægypti* (argenteus), formerly known as *Stegomyia fasciata*. Infection is only possible if the insect has fed from a patient in the first three days of the disease, but the insect does not become infective to



FIG. 85.—YELLOW FEVER INCLUSION BODY (HUMAN LIVER) ( $\times 1000$ ).

1. Peripheral masses of chromatin.
2. Irregular granular ring-form of Inclusion Body surrounding a central mass of chromatin.

another individual for at least four days, and not for a much longer time, if air temperature is low. Infection may be conveyed directly from man to man by contact with the blood or serum of a patient. Filtration of the serum through a Berkefeld or one of the coarser Chamberland filters does not render it incapable of causing infection. The virus particles measure from 17 to 28m $\mu$  in diameter.

When the mode of transfer of the virus of the disease from

man to man by *Aedes ægypti* was discovered, it was hoped that the disease would be rapidly stamped out. This was actually done in civilized communities, but a form of yellow fever lingers in Brazil. This is known as jungle or silvatic yellow fever. The reservoirs of the virus are monkeys, but the transmitting insect has not yet been identified with certainty.

Investigation of the disease was hampered by the fact that the majority of laboratory animals are insusceptible but, in Lagos in 1927, it was discovered by Stokes and other members of a Rockefeller Commission that a monkey, *Macacus rhesus*, could be infected. The virus, although unseen and uncultivated, was carried through many monkeys, either by direct passage or through the intermediary of the mosquito. Later other animals, mice and hedgehogs for example, were found to be susceptible to infection.

By intracerebral inoculation of mice in series, the virus becomes neurotropic and loses its viscerotropic character. It has then lost much of its virulence for man, and a virus so modified has been used either alone or in conjunction with convalescent human or immune monkey serum to confer active immunity. A pantropic virus, grown in a tissue culture of chick embryo with the central nervous system removed, has also been extensively used. Passive immunity can be produced by convalescent serum which is actively antiviral.

Since the serum of a person who has recovered from yellow fever continues to contain antiviral bodies for years, the extent of which the disease is or was formerly prevalent in a community may be ascertained by testing the sera of a number of persons for antiviral properties. This is done by the injection of a mouse with serum and mouse-brain virus. If the mouse survives, the serum contains protective substances indicating recovery from yellow fever. In this way "silent areas" have been discovered, where no typical cases of yellow fever had been known to occur, but where the disease either exists in atypical form or has previously existed.

In the liver cells both of man and of experimental animals, very characteristic inclusion bodies are found.

Since it is known that the disease is transmitted, under

natural conditions, mainly by the bite of *Aedes ægypti*, it is obvious that the disease can be exterminated by destruction of that mosquito, and this has been largely accomplished in the American continent except in Brazil: conditions in Africa render such control less easy.

It is not inappropriate to record that knowledge of the facts concerning Yellow fever has been obtained only by sacrifice: Lazear in America in 1900, and Stokes, Noguchi and Young in West Africa in 1927 died of Yellow fever when investigating the disease.

### **Influenza.**

Influenza is due to a virus which measures from 80 to 120m $\mu$ . It is possible to infect ferrets by intranasal inoculation of material from a human case. The animal develops a characteristic febrile disease and, in some cases, pneumonia occurs. After ferret to ferret passage, the virus is capable of affecting mice.

There are several serologically distinct strains of the virus, one of which is very similar to the virus of swine influenza, and it is possible that this latter virus may be pathogenic for man.

An animal recovered from the disease possesses considerable immunity and antiviral bodies are present in its blood serum.

Active immunity may be produced in susceptible animals and man, and, for this purpose, killed virus appears to be almost as useful as living. Suitable vaccines may be prepared from the lungs of infected mice and from chick-embryo cultures treated with formalin. It is possible to demonstrate the presence of antiviral bodies by complement fixation in the blood serum of an immunized man, but it has not yet been proved that this process of immunization protects against the natural disease.

### **Psittacosis.**

Psittacosis is a naturally occurring disease of birds, particularly of parrots, parakeets, budgerigars and fulmar petrels which is transmissible to man. In the bird it causes diarrhoea,

sometimes accompanied by pneumonia. Not infrequently birds act as healthy carriers of the virus. Infective birds, whether sick or carriers, are usually those recently imported from South America, where the disease is enzootic, or wild sea-birds caught for food, as in the Faroe Islands.

Human beings are infected from the faeces of birds. Man to man spread is very rare. Psittacosis in man affects chiefly the lungs, in which pneumonia occurs. The fatality rate is about 20 per cent.

Intraperitoneal inoculation of mice with sputum from a human case or with filtered suspensions of the spleen or liver from an avian case causes a characteristic fatal illness which is of value in confirming the diagnosis.

The virus can be cultivated on the chorio-allantoic membrane of the developing chick.

### Other Diseases due to Viruses.

Among other human diseases which are commonly believed to be due to viruses are measles, common cold, mumps, and dengue.

The virus of measles is present in the blood and nasopharyngeal washings, and is transmissible to monkeys and rabbits. It can pass through a Berkefeld filter. Some believe that the cause is a diplococcus or streptococcus, but the virus theory is more probable. Intramuscular injection of 2 c.cs. of convalescent serum for each year of age within the first five days of incubation, usually prevents the disease; later the disease may be modified by such an injection. Adult serum is less effective but, in double the dose indicated above, has considerable value in attenuating the disease. A solution of the globulins extracted from the blood contained in human placenta has also been used to confer passive immunity against measles. It appears to have an efficiency similar to that of adult serum.

Little is known about the virus of Mumps except that it is present in the saliva.

Dengue is an example of a virus disease spread by an insect. The virus, which is filterable, is present in the blood,

and the disease can be transferred from man to man by the injection of blood. Under natural conditions a mosquito, *Aedes ægypti*, acquires the virus from a man in the first few days of the disease and, after about eight days, becomes infective.

Many animal and plant diseases which cannot here be referred to in detail are due to viruses. If the theory of d'Herelle and many others is correct, bacteria can suffer from infection with a virus—the bacteriophage—which, it must be confessed, in many respects behaves like a virus.

Certain types of new growth, most usually regarded as malignant (*e.g.* Rous sarcoma), can be initiated by inoculation of a healthy animal with filtrates of extracts of the tumour. It is fairly generally believed that a filterable virus is the cause of production of these tumours. Whether a virus plays any part in the causation of other malignant tumours in man remains *sub judice*.

#### Diseases due to *Rickettsiæ*.

Epidemic typhus fever is a disease closely associated with filthy conditions. It is characterized by high fever and an extensive petechial eruption. The fever usually terminates about the fourteenth day, when the crisis occurs. The fatality rate, which is very high in those over middle age, may vary from 10 to 25 per cent.

The chief post-mortem findings are a peri-vascular accumulation of mononuclear leucocytes and a proliferation and degeneration of the endothelium of the smaller vessels, usually accompanied by thrombosis.

The disease may be communicated to the anthropoid apes, smaller monkeys and guinea-pigs by injection of the blood or a suspension of organs of a patient. In the former animals, an eruption, resembling that seen in the case of the disease in man, appears. In the guinea-pig the disease is an acute fever which is rarely fatal. The virus may be passed successfully through a long series of these animals. Infection of animals is most easily produced by intraperitoneal injection of the blood of a patient.

In the blood, and in the endothelial cells of vessels, small



rod-like or egg-shaped bodies, measuring from 0.3 to 0.5 $\mu$  (*Rickettsia prowazeki*), are found and there are very strong grounds for regarding them as causal. They have not been cultivated apart from living cells and are not filterable.

Epidemic typhus is transmitted by the body louse and it is found that the insect becomes infective from 2 to 11 days after feeding on the patient. In the gut of the louse, bodies are seen apparently identical with the *Rickettsia* bodies. These are very numerous in the epithelial cells of the gut, and although somewhat similar bodies are found in lice which have not fed on typhus patients, they are never found intracellularly. Animals can be infected by allowing lice, which have previously fed on human cases, to bite them, or by injection of fæces from an infected louse; and in their bodies *Rickettsiæ* are found.

One attack of the disease confers immunity, and the serum of men or animals, convalescent from the disease, is capable of destroying the virus and may be used to confer passive immunity. Prophylactic vaccines, made by heating to 55° C. the blood of an infected guinea-pig or a suspension prepared from the intestines of infected lice have been used, but a phenolized vaccine prepared from a culture of the *rickettsiæ*, grown in a tissue culture of guinea-pig tunica vaginalis, is preferable and, it is claimed, gives good results.

Wilson found that the serum of a typhus patient had the power of agglutinating certain intestinal bacilli, and in the Weil-Felix test the property which the serum has of agglutinating. *Proteus* X19 is made use of as an aid to diagnosis. It is almost invariably found that the serum, after about a week's illness, agglutinates the bacillus in a dilution of  $\frac{1}{50}$  or more, and at a later period its titre may exceed  $\frac{1}{2000}$ . Agglutination is always of the O type.

In addition to epidemic typhus, endemic typhus of a number of different types exists in various parts of the world. The chief of these is the murine type which is spread from the rat, which acts as reservoir of the causative *Rickettsiæ*, to man by the rat flea (*Xenopsylla cheopis*). Since infected rats do not die of the disease, they are not deserted by their fleas,

and so epidemics of the disease do not occur in man. Brill's disease is probably of this type. Another type of typhus (Rocky Mountain Spotted Fever) is spread by a tick (*Dermacentor*), and yet another (Tsutsugamushi) by a mite (*Trombicula*). It is probable that animal hosts, other than man and rat, exist for flea, mite and tick borne typhus. The relationship between the various forms of typhus is shown by the presence in each of *Rickettsiæ* and the practically constant agglutination of a proteus by the sera of patients. *Proteus* X19 is not, however, agglutinated in all types of typhus. In some, agglutination is best shown with strain X2 and in others with strain X Kingsbury. Some workers believe that *Rickettsiæ* are derived from a proteus, but this is, as yet, unproved.

Trench fever is a disease bearing a certain resemblance to typhus fever, but of a much milder type and with practically no mortality. It is conveyed by lice, and in the body of an infected louse organisms which are probably the cause of the disease have been found. These (*Rickettsia quintana*), are very similar to the *Rickettsia* of typhus fever. They never occupy an intracellular position and never occur in chains, characteristics which distinguish them from *R. prowazeki*.

## CHAPTER XLV

### PATHOGENIC PROTOZOA

#### **Malaria.**

MALARIA is a disease chiefly of tropical or sub-tropical countries although it is not unknown in temperate climates and was, until fairly recently, common in certain parts of England and Ireland. It is characterized by attacks of fever which recur at more or less regular intervals for some considerable time. Anæmia and enlargement of the spleen are almost always observable.

The disease is due to the presence in the red blood corpuscles of minute protozoa, the malaria parasites, of which three distinct races are known. These can be distinguished by their microscopic appearance and also by the type of disease produced. Benign tertian malaria is due to the *Plasmodium vivax*; Quartan malaria is due to the *Plasmodium malariae*; and Malignant or Sub-tertian malaria to *Plasmodium falciparum*. Malaria may be conveyed by the injection of blood from a patient into a healthy individual, as has occurred in blood transfusion, but in nature the disease is transmitted exclusively by the bite of a female mosquito of the genus *Anopheles*. In the human body the parasites multiply asexually, while sexual multiplication occurs in the mosquito. For this reason the mosquito is regarded as the definite host of the protozoon and man as the intermediate host.

The life history of one of the parasites, *Plasmodium malariae*, will first be described, and subsequently the characteristics which distinguish it from those causing the other types of malaria will be mentioned. When the mosquito bites, she

injects into the human body a small amount of saliva which may contain a large number of young parasites. Each of these is a minute, thin, pointed rod, having about its centre a nucleus, easily distinguished in stained preparations by its chromatin. This, the Sporozoite, penetrates a red blood cell and becomes the Trophozoite or Amœbula. While still young, the trophozoite is commonly found as a ring-shaped body which appears to enclose some of the hæmoglobin of the cell. The ring resembles a signet ring, since it is thicker on one side than the other, and the nucleus is usually situated in the thinner side. The trophozoite grows at the expense of the hæmoglobin of the cell and frequently assumes an oval or band-like shape. Waste material accumulates in its body in the form of coarse grains of a dark brown pigment, scattered chiefly towards its edge, the nucleus now being situated centrally. When examined alive at this stage its outline is found to be constantly altering within the red cell; in fact, it behaves very much like an ordinary amœba. The fully mature amœbula, now the Schizont, almost fills the red cell, which otherwise does not appear to be greatly altered. Various activities take place; the nucleus breaks up into a number of fragments which scatter through the protoplasm, while the pigment accumulates in the centre. After the division of the nucleus the protoplasm also divides, and the parasite is now found to have produced from 6 to 12 new parasites, the Merozoites, which are regularly arranged like the petals of a daisy. A certain amount of residual protoplasm surrounds the central pigment. The red cell disintegrates and the brood of merozoites is free in the plasma and, at the same time, the pigment is also liberated. A merozoite approaches a fresh red cell, penetrates it and develops just as did the original sporozoite, only to divide in course of time into yet more merozoites. The process of multiplication by asexual division in the human body is known as Schizogony. In the case of quartan fever the cycle is one of 72 hours (A paroxysm of fever recurs on the fourth day, counting the day of a bout as one.) If only one sporozoite has been injected by the mosquito it will have produced, say, 10 merozoites at the end of 72 hours; after a

further 72 hours there will be 100, and so on. The parasites adhere fairly rigidly to their time-table, so that at any moment the majority are at exactly the same stage of development, a few being slightly too rapid and a few too slow. The setting free of all the merozoites in the patient's blood, therefore, occurs at about the same time, and it is found that the fever commences at this time. The cause of the fever is certainly some toxic substance, either the pigment or some other waste product, liberated when the red cells disintegrate and the merozoites escape. It has been found that if the serum obtained from the blood of a patient during a paroxysm be filtered and injected into a healthy man it gives rise to a single bout of fever similar to that of malaria.

The disease has an incubation period of about a fortnight, the time necessary for the development of sufficient parasites in the blood. It has been calculated that for the production of symptoms there must be several hundred parasites in each cubic millimetre of blood. From this time onward, in the absence of treatment, bouts of fever occur at intervals of about 72 hours. If, however, the patient has been infected on two occasions, the two broods of parasites resulting from the injected sporozoites will each follow its own time-table, and schizogony of each brood will occur every third day; but the unfortunate patient will experience two bouts of fever every three days. In the same way, as the result of three distinct infections, fever may occur every day. Rarely does the mosquito inject a single sporozoite; but however many are injected, the descendants of those injected at the same time all follow the same time-table, the only difference being that a slightly longer time will elapse before the first attack of fever where only one was injected than when the number was large.

Simultaneously with the production of merozoites two other types of cell develop in the patient's blood in preparation for the sexual cycle and sporogony, in the mosquito. The development of these resembles that of the ordinary trophozoite, but no division of the nuclear material occurs. These, the gametocytes, enlarge and become rounded. The Macrogametocyte (female) is larger, takes stain more intensely, and

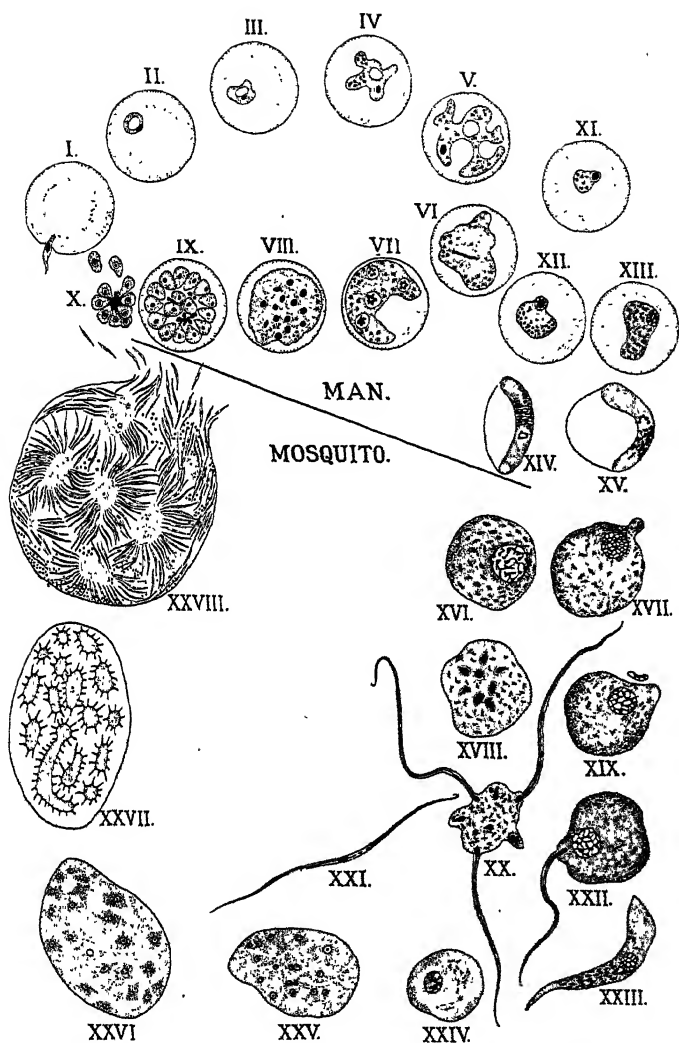


FIG. 86.—DIAGRAM OF THE DEVELOPMENT OF THE MALARIA PARASITE.

I, Sporozoite. II to VIII, XI, Trophozoite. IX, Schizont. X, Merozoites. XII, XIV, XVI, XVIII, Microgametocyte. XIII, XV, XVII, Macrogametocyte. XX, XXI, Microgamete. XIX, Macrogamete. XXII, Zygote. XXIII, Oökinete. XXIV, XXV, XXVI, Oöcyst. XXVII, Oöcyst with Sporoblasts. XXVIII, Oöcyst with Sporozoites.

(Note.—XXVI to XXVIII are shown less highly magnified than the other drawings.)

has more pigment in coarse grains arranged in a circle around its centre, while the Microgametocyte (male) is smaller, stains (with Leishman's stain) a grey-blue colour, has less and finer pigment, less regularly distributed, but a larger nucleus. These cells do not develop further in the human body; but if a drop of blood be examined on a warm stage, the macrogametocyte matures by the extrusion of a portion of its nucleus and becomes the Macrogamete. The pigment in the interior of the microgametocyte becomes violently agitated and dances. Suddenly a number, from one to six, of long delicate, flagella-like



FIG. 87.—MACROGAMETOCYTE OF  
*P. FALCIPARUM* ( $\times 1200$ ).

structures, 15 to 20 $\mu$  in length, shoot out and lash vigorously. These, which are the Microgametes, have a fine core of chromatin and a slightly bulbous end. They break free from the remains of the gametocyte and swim until they come in contact with a macrogamete. One impregnates this, the nuclear materials fuse and the result is the Zygote. This process never takes place in the blood in the body, and

normally only in the stomach of a mosquito which has fed from a human patient. The zygote becomes oval, elongated and, as the Oökinete, almost wormlike. It penetrates the wall of the stomach and lodges between the muscle fibres, and again becomes a rounded body of about 6 $\mu$  in diameter. It grows rapidly, surrounds itself with a capsule and becomes the Oöcyst. The oöcyst soon comes to form a large swelling, protruding from the wall of the stomach into the body cavity of the mosquito. The nucleus and protoplasm divide and a number of Sporoblasts are formed, each of which divides into a large number of thin spindle-shaped, nucleated cells, arranged

radially. The sporoblasts disappear and these threads, the Sporozoites, are free within the capsule, which may now have a diameter of  $60\mu$ . This bursts and the sporozoites find themselves in the insect's body cavity, from which they make their way to the salivary glands and are injected by the insect into the next human being on whom she feeds, there to set up the schizogony cycle again. The sporogony cycle occupies on an average from ten to twelve days.

In *Plasmodium vivax* schizogony is very similar. The amoeboid movement of the amoeba is, however, much more active. The parasite tends to be rather larger and the red cell is pale and swollen and frequently shows, when stained, deeply stained points, Schüffner's dots. The pigment within the parasite is finer and of a lighter colour. In schizogony from 15 to 20 oval merozoites are formed, which are arranged regularly in the form of a rosette. Schizogony occurs every forty-eight hours. Since it is of a relatively mild type, this form of the disease is called Benign tertian malaria. Sporogony does not differ in essentials from that in the quartan parasite.

Malignant or Sub-tertian malaria is of a more serious character. The occurrence of the fever is less regular, although mainly every forty-eight hours, and multiple infections are common. The young amoebulae are smaller and show more active amoeboid movements than those of *P. malariae*. When fully developed the schizont occupies less than half the red cell, which appears shrunken and deeper in colour than normal. Pigment is scanty and occurs in fine grains. From 6 to 20 merozoites, irregularly arranged, are produced, but this stage is very rarely seen in the peripheral blood, as the second twenty-four hours of the cycle, including schizogony, is confined almost exclusively to the internal organs, particularly the spleen. The gametocytes differ from those of the other two parasites since they are of crescentic or sausage shape. They are larger than the cells in which they developed, the remains of which may sometimes be observed as a fine line uniting the points of the crescent like the string in a bow. The macrogametocyte is larger, has pointed ends, stains more intensely, has a smaller nucleus, and the pigment is collected towards the



centre. In the microgametocyte, which has rounded ends, the pigment is distributed through the protoplasm of the parasite. Outside the body the gametocytes lose their crescentic outline, become rounded, and further development is very similar to that of *P. malariae*.

The laboratory diagnosis of malaria depends on the finding of the parasite in the blood. Three methods are employed: in the first a fresh thin film of blood is examined unstained; the second is to make blood films in the ordinary way and to stain with Leishman's or some other stain; the third, the thick-film method of Ross, is employed where parasites are few. About 20 c.mms. of blood are spread over an area of about one square inch and allowed to dry. The film is inverted in a few drops of a  $\frac{1}{15}$  dilution of Giemsa stain and left there for 20 minutes. It is then washed, dried and examined. Some experience is needed before the observer can rely on finding all stages of the parasite when present, and on distinguishing platelets and artefacts, such as deposited stain, from parasites. A prolonged search may be necessary before even a single parasite is found. In the absence of parasites, the presence of an excess of large mononuclear leucocytes in the blood and of pigment in the leucocytes may assist in establishing a diagnosis.

It has been found possible to cultivate the malaria parasite in defibrinated blood containing 0.5 per cent. glucose at a temperature of 40° to 41° C. Schizogony proceeds within the red cells, but sporogony does not occur.

The chief symptoms of malaria are due to the toxic products of the parasite which are thrown into the blood stream at schizogony. In addition, anæmia is present, and the number of red cells is diminished to an extent which cannot be explained by the number infected. There appears to be a definite destruction of the cells by hæmolysis, due either to the pigment or other toxic product. Leucopenia is usually to be observed, but there is an increase in the number of large mononuclear cells. The most obvious pathological change is the deposition of the pigment in the internal organs, especially in the spleen, liver, bone marrow and brain. The pigment is found chiefly in the endothelial cells of the capillaries,

which, in consequence, may degenerate, leading to capillary hæmorrhages; but in the spleen and bone marrow it is found all through the tissues.

It is well known that in malaria the patient may have remissions, during which no attacks of fever are experienced; but after a longer or shorter time typical bouts of fever recur, often as a result of a chill or some indiscretion. The explanation is probably that a certain degree of immunity is developed, and that the parasites live and go through schizogony in small numbers, possibly in some internal organ. It is only when a large number of red cells are attacked that fever develops. Very little real immunity is established against the parasite, but there may be some immunity against the toxic substances produced. Certainly negroes are less affected by malaria than are Europeans.

Quinine has for long been regarded as the most effective agent in the treatment of malaria, and its free administration appears to destroy the great majority of the asexual parasites. It is, however, without effect on the gametocytes. Recently plasmoquin and atebirin have been introduced for the prevention and treatment of malaria and very good results are reported, particularly in the control of gametocytes.

In the prophylaxis of malaria there are three cardinal factors: (1) Malaria is spread by the bites of mosquitoes; (2) mosquitoes bite at night; (3) mosquito bites are only dangerous if the insect has previously fed from an infected human being. The first necessity is to set about eradicating the mosquito. Since water is necessary for the development of the larva, all stagnant water should be drained or, where this is impossible, covered with oil, which prevents the development of the larva. While mosquitoes still exist quinine should be administered to all those already infected in order to keep the number of parasites in their blood as low as possible. Europeans should sleep some distance away from infected natives, for the mosquito does not fly far. At night, the house or bed should be rendered mosquito-proof by wire or other netting. There is some discussion as to the value of a daily small dose of quinine in preventing the onset of the

disease, but it seems fairly well established that some advantage is conferred by it.

### Amœbic Dysentery.

Amœbic dysentery is a disease quite distinct from that due to the presence of the dysentery bacilli. The onset is more gradual, there is but little fever, and the number of motions passed is few but, as in the bacillary disease, they contain both mucus and blood. The disease is inclined to be chronic rather than acute, the patient becomes more and more emaciated, and in about 25 per cent. of cases not suitably treated death occurs. The sporadic form of amœbic dysentery is common in tropical and sub-tropical countries ; epidemics are rarely experienced.

The organism, the *Entamoeba histolytica*, is found in the fæces, in the ulcers of the intestine and in the abscesses of the liver which are a frequent complication of the disease. If a fluid stool from a patient be examined fresh, the amœba is seen to measure from 20 to 30 $\mu$  in diameter. It is of a faint green colour, and can be easily found owing to its high refractivity. The outer zone, the ectoplasm, is absolutely clear and glass-like while the central part, the endoplasm, is slightly granular. In the interior may be distinguished the delicate, highly refractile nucleus, which is usually placed eccentrically, and also one or more vacuoles and various food substances, chiefly red blood cells and bacteria. The outline of the organism is constantly changing, owing to the rapid emergence of blunt pseudopodia in which the distinction between ectoplasm and endoplasm is easily seen. The amœba moves actively by a flowing motion. Its minute structures may better be observed in preparations which have been fixed while still wet in hot alcoholic corrosive sublimate solution and stained with iron-hæmatoxylin. The nucleus is found to have a definite, sharp, circular or oval outline, with a central karyosome and a small amount of chromatin arranged in dots on the inner surface of its membrane. In the later stage of the disease, and during convalescence, the form of the *E. histolytica* described above may be seen only rarely or not at all, since the entamœbæ

have become encysted. The cyst, which is much smaller than the vegetative form, measuring from 10 to 15 $\mu$  in diameter, is round, and has a definite outline with double contoured wall. In its interior neither blood cells nor vacuoles are seen, but four rounded nuclei with sharp nuclear membranes studded with chromatin masses may be found. In the unstained film these are not seen with any great ease, but the addition of a small amount of Gram's iodine facilitates their detection. In many cysts, probably those not yet mature, one or more elongated chromatin masses, the chromidial bodies, may be seen.

As the diagnosis of the disease is made by the identification of the parasite in the stools, it is of great importance to distinguish it from another very similar organism, the *Entamoeba coli*, which is frequently found and which is non-pathogenic. This is usually somewhat larger than *E. histolytica*. In it the distinction between ectoplasm and endoplasm is not definite,

the nucleus is much more easily seen, has a larger amount of chromatin, and is situated near the centre, the endoplasm is less granular, vacuoles are fewer and ingested red cells are rare, but bacteria are common. The cysts, which are from 15 to 20 $\mu$  in diameter contain 8 (rarely 4 to 16) nuclei and frequently a vacuole. The organisms (*E. coli* as well as *E. histolytica*) multiply by binary fission. The cysts are a resting stage in which the protozoa, in a slightly more resistant form,

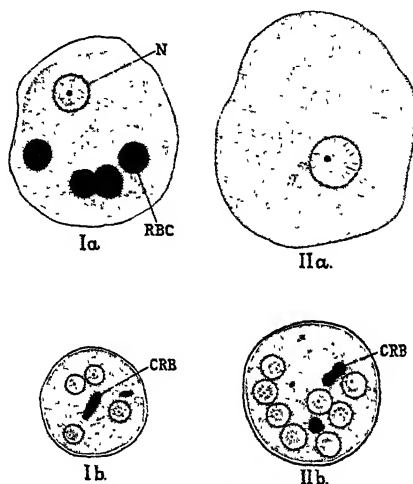


FIG. 88.—ENTAMOEBAE.

Ia. *E. histolytica*: N, nucleus; RBC, red blood cells. Ib. *E. histolytica*, cyst; CRB, chromidial body. IIa. *E. coli*. IIb. *E. coli*, cyst.

may have a better chance of reaching their natural habitat, the intestinal tract of a fresh victim. For this reason cysts are produced chiefly towards the end of the disease, at which period very few amœbæ may be found in the stools. When swallowed, the amœbæ are destroyed by the gastric juice, but the cysts escape and reach the intestine. There they may settle down and multiply and act merely as saprophytes (*E. coli*), or either as saprophytes or pathogens (*E. histolytica*), for the latter organism does not in every case produce dysentery. In an experiment 20 men were given *E. histolytica* cysts by the mouth. In 18 the amœbæ were found in the stools, but only in four were the symptoms of dysentery noted. The possibility of this organism existing in the intestine for long periods without dysentery resulting explains the finding of it in stools of individuals who have never had dysentery, and is also probably of importance in the spread of the disease, since carriers may be either healthy or convalescent.

In the disease in man the organism makes its way between the epithelial cells and so into the sub-mucosa of the large intestine, especially in the region of the cæcum and at the flexures of the colon. A gelatinous œdema results, with little leucocytic invasion of the part. Necrosis occurs, the slough separates off and an ulcer is produced. This may be from microscopic dimensions up to 3 cms. in diameter. It has irregular, overhanging edges and a ragged floor composed of the muscle coat. Amœbæ may be found in the ulcer, particularly in the edges beyond the ulcerated area. The parasite may be carried in the blood or lymph to other parts of the body, most commonly the liver, where it frequently produces the so-called tropical abscess, of which one or more may be present. These are of irregular outline with ragged edges, and the contents, which are thick, slimy and of a chocolate or pinkish colour, consist chiefly of necrosed liver tissue and blood with few pus cells. Large numbers of amœbæ are found in material obtained by scraping the wall of the abscess. They are entirely of the vegetative form ; cysts are not present.

The diagnosis of amœbic dysentery is made by finding either the characteristic amœbæ or cysts in the stools. The

stool should be examined as fresh as possible in the wet state, films being made from a portion of blood-stained mucus when available, both with and without the addition of Gram's iodine.

The disease is spread by the faeces of a patient or carrier, and infection may occur from water or food, particularly uncooked vegetables. Flies are probably important mechanical agents in transmitting amœbæ.

Dysentery may be produced in young cats and dogs by feeding with material containing the cysts of the *E. histolytica* or, more certainly, by the injection of such material into the rectum.

Cultures of some of the non-pathogenic entamœbæ have been made on a variety of media, but it is only within the past few years that *E. histolytica* has been cultivated. This has been accomplished by Boeck and Drbohlav using a medium consisting of solidified egg covered with dilute human serum. The cultivated entamœbæ exhibit characteristic pathogenicity for kittens.

Emetine is extensively used for the treatment of the disease with, in the majority of cases, successful results, and good reports have been received as to the utility of "Yatren 105."

### Trypanosomes.

The trypanosomes are motile protozoa, living in the blood plasma of man or of the lower animals, and producing in these a variety of diseases. Minor differences characterize each species, but fundamentally they are very similar. A trypanosome has an elongated body, measuring from 15 to 30  $\mu$  in length by 1.5 to 2.5  $\mu$  in breadth in the thickest part, from which it tapers towards each extremity. At one pole, the anterior, is a long, fine flagellum which commences near the posterior end in the blepharoplast, close to the kinetonucleus, and is continued anteriorly along the edge of a delicate, indented, fin-like structure, the undulating membrane. In its interior, near the centre

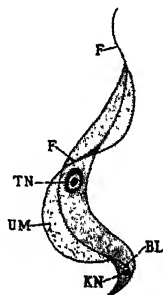


FIG. 89.—DIAGRAM OF TRYPANOSOME.  
F, Flagellum. TN, Trophonucleus.  
UM, Undulating membrane. BL, Blepharoplast.  
KN, Kinetonucleus.

in the stained specimen, is seen a round or oval body (the macronucleus or trophonucleus) while near the posterior pole is a much smaller structure, the kinetonucleus. The organism swims actively through the plasma, disturbing the blood cells as it goes. Its motility appears to be due to the flagellum and to contractions of its body, but chiefly to the undulating membrane. In the vertebrate body multiplication takes place by longitudinal division. The two resulting trypanosomes may be equal in size, but commonly one is very much smaller than the other. In the same blood considerable variations

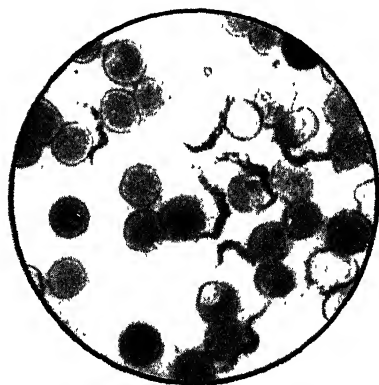


FIG. 90.—*TRYPANOSOMA EVANSI* IN RAT BLOOD ( $\times 950$ ).

in the parasites may be found, and these have been regarded by some as male and female forms. Sexual reproduction has not been observed in the vertebrate body, but it may occur in the other host, usually an insect. It is almost certain that in the latter some developmental process takes place.

In man the chief disease due to trypanosomes is sleeping sickness, one of the most serious of the diseases of tropical Africa. The disease may be divided into two stages, which were formerly believed to be quite distinct conditions. The first, trypanosome fever, was ascribed to the *Trypanosoma gambiense*, while to the parasite found in the second stage, sleeping sickness, the name *Tr. ugandense* was given. It is now generally believed that sleeping sickness is but a later stage of trypanosome fever, and that *Tr. gambiense* is identical with *Tr. ugandense*. Since the former of these was first described, the name of the organism found in either condition is *Tr. gambiense*.

The disease commences with irregular fever, wasting, muscular weakness, enlargement of the spleen, and the

occurrence of areas of local erythema and oedema. The condition is rather chronic, but may cause death. If death does not occur at this stage, after a longer or shorter time, a change in disposition is observed. The patient becomes morose and apathetic; the speech is slow and headache and indefinite pains are complained of; the superficial lymphatic glands are enlarged; muscular tremors occur, and there is progressive emaciation and anæmia; the lethargy becomes more marked and passes into coma, followed by death. The total duration of the disease may be several years. The chief pathological changes are congestion and inflammation of the meninges and, in the brain, a perivascular infiltration with lymphocytes.

In the early stages of the disease the trypanosomes are found in the blood, but not in large numbers. Later they are present in the cerebro-spinal fluid and in the juice of the enlarged glands.

Injection of material containing the parasites into monkeys gives rise to a disease closely resembling that of man. Dogs, cats, and white mice are also susceptible, and guinea-pigs to a much less extent.

The *Tr. gambiense* has been cultivated on the rich blood-agar medium of Novy and MacNeill at 22° C. A similar medium has been found useful for the cultivation of other trypanosomes.

The disease is spread by the bite of *Glossina palpalis* (the tsetse fly), of which both male and female bite in daylight. This insect may transmit the disease mechanically for a short time after the infecting feed. Then, for a period of several weeks, a bite is without effect, but subsequently to this, biting may reproduce the disease and the insect may remain infective for a considerable time. From this it seems very likely that some cycle of development occurs in the insect, but the full details are not yet certain. There are strong grounds for believing that the *Tr. gambiense* can live in the blood of wild animals, and it appears probable that the disease is kept alive in a district by antelopes and other big game acting as reservoirs of the organism.

Animals may, to some extent, be immunized against this trypanosome and their sera are found to destroy the organism



and to protect other animals against infection. Serum useful for the treatment of the disease in man has, however, not yet been produced.

Sleeping sickness, when fully developed, is always fatal if untreated, but the earlier manifestations may become arrested and the lethargic stage may not be reached. Many drugs have been tried, of which the most promising until recently were certain arsenical derivatives (atoxyl, salvarsan, etc.) and salts of antimony. These undoubtedly are very effective in reducing the number of parasites in the blood, and can produce cures in experimentally infected animals. A certain number of human infections appear to have been definitely cured, but success has not usually attended the use of these drugs once the central nervous system has been attacked. A few apparent cures have, however, been reported following the use of tryparsamide in cases in which the central nervous system was involved. A drug of unrevealed formula, but stated to contain neither mercury nor arsenic, Germanin ("Bayer 205"), has been used with apparently good results in a large number of cases, especially in the early stages of infection with *Tr. gambiense*.

One great difficulty and danger in the treatment of diseases due to trypanosomes is that, if the patient receives inadequate doses of a drug, his trypanosomes may become drug-resistant so that they are unaffected by a concentration of the drug lethal to normal trypanosomes. This resistance is usually called arsenic-fastness, since it was first observed with arsenical preparations. It is not, however, a resistance to arsenic, but to the substituted phenyl radicals of the aromatic compounds used. Resistance is maintained for many generations of the trypanosome and has been found still present after five years, during which the organisms were passaged through hundreds of mice and also tsetse flies. There are reasons for believing that, in parts of Africa, resistant strains are becoming increasingly common as the cause of human disease. It is, therefore, important in the interests both of the patient and of the community, that dosage should be adequate, especially in the case of aromatic arsenical and antimonial compounds.

*Tr. rhodesiense* is an organism almost identical with the *Tr. gambiense*, but causing in man a more rapid and virulent form of the disease. As a result of immunization experiments in animals it is found to be a distinct organism serologically. In the body of the rat it may be distinguished from the *Tr. gambiense* by the fact that the trophonucleus may occasionally be situated posteriorly to the kinetonucleus. Many experienced workers believe that *Tr. rhodesiense* is the same organism as *Tr. brucei*, the cause of nagana in horses, possibly modified by passage through man. It certainly is transmitted by the same fly, *Glossina morsitans*.

A considerable number of trypanosomes are found as parasites in other animals. The most widespread of these is *Tr. lewisi*, found in the blood of rats in various parts of the world. The animal seems to be little injured by the presence of the organism and to acquire immunity to it after a few months, when the blood is found to be free of trypanosomes. The parasite is probably transmitted by the rat flea or louse, and possibly infection can occur by the intestinal route.

Nagana, a very fatal disease affecting animals in various parts of Africa, is due to *Tr. brucei* (horses and dogs), *Tr. congolense* or *Tr. vivax* (cattle). The disease is conveyed by a tsetse fly, *Glossina morsitans*, and the reservoirs from which the flies are infected are the antelopes and other wild animals of the part, which are apparently unaffected by the presence of the parasites. *Tr. evansi* also affects horses, producing in India the disease known as surra.

*Tr. cruzi*, a trypanosome which differs considerably in morphology from those described above, is the cause of Chagas' disease, which occurs in Brazil and other parts of South America. The parasite is transmitted by a bug, *Conorrhinus megistus*.

Diagnosis of trypanosomiasis is usually made by the finding of the trypanosome in blood, cerebro-spinal fluid, or in gland juice. In the case of blood infections, the fluid should be diluted with saline and examined as a wet preparation. Films should also be prepared and stained by Leishman's

stain. If the number of parasites is small, the centrifuging of citrated blood and examination of the upper layer of deposited cells may facilitate the search. Cerebro-spinal fluid usually requires prolonged centrifuging. Inoculation of animals—preferably monkeys, dogs or rats—with suspected material and the subsequent finding of the parasite in the blood may occasionally enable a diagnosis to be made.

The control of the diseases caused by trypanosomes requires the extinction or at least the reduction of the transmitting flies, and possibly the extermination of the wild animals which act as the reservoirs of the parasites. It has been found that 1.0 to 1.5 gm. of Germanin injected intravenously renders a man insusceptible to infection by trypanosomes for more than three months.

### Leishmaniasis

Kala azar, known also as Dum-dum fever and by many other names, is a fairly common disease occurring in India, China, Malay and in many other tropical countries, either sporadically or, more rarely, in epidemic form. It is a chronic disease in which the rate of mortality in untreated cases is very high. Its outstanding features are irregular fever, emaciation, anæmia, enlargement of the spleen and liver, local œdema, and the occurrence of ulcers both of the skin and intestine, especially the colon.

The causal organism (the *Leishmania donovani* or Leishman-Donovan body) is found in enormous numbers in films made from the spleen or liver. It is most usually oval or of cockle-shell shape, from 2 to 4 $\mu$  in greatest diameter. When stained with Leishman's stain it is of a faint blue colour, and in the interior are to be seen two bodies, the larger oval or irregular, the trophonucleus, and the smaller, situated close to the former, a rod-shaped body, the kinetonucleus, both of which take a pink colour. One or more vacuoles may also be distinguished. The parasites are most usually seen in the interiors of large mononuclear (endothelial) cells. They multiply by simple fission until the host cell is distended and bursts, liberating them, when they invade new cells. The Leishman-Donovan

bodies are most frequently seen in the spleen, liver and bone marrow and, in smaller numbers, in the lung, kidney, lymphatic glands and ulcers of the skin and intestine, almost always in the endothelial cells of blood vessels and lymphatics. They may also be found, after prolonged search in many cases, in the peripheral blood, usually in either mononuclear or polymorphonuclear leucocytes.

The organism can be cultivated on the medium of Novy and MacNeill used for trypanosome culture. On this medium

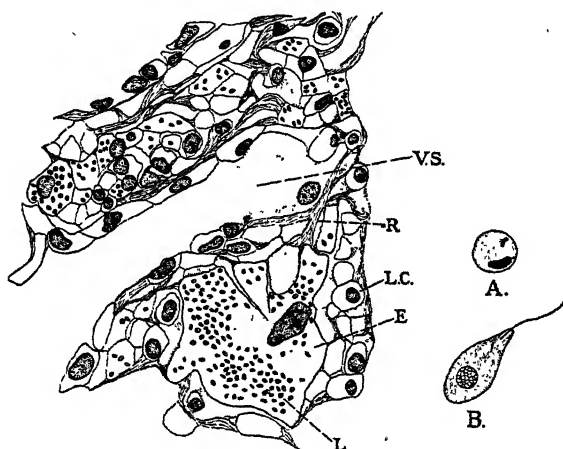


FIG. 91.—*LEISHMANIA DONOVANI* IN SECTION OF SPLEEN.

V.S. Venous sinus. L.C. Lymphoid cell. R. Reticulum.  
E. Large endothelial phagocytic cell. L. *L. donovani*.  
A. *L. donovani* (greatly enlarged). B. *Herpetomonas*  
form of *L. donovani* (greatly enlarged).

the organism enlarges, becomes elongated and develops a flagellum (herpetomonas form). It then bears some resemblance to a trypanosome, but no undulating membrane is present.

The organism is pathogenic for monkeys, rats and mice and, to a less extent, for dogs, guinea-pigs and rabbits. Infection is most certainly produced by intraperitoneal or intrahepatic injection of large amounts of an emulsion of

infected tissue or of culture. In a few experiments, monkeys have been infected by feeding. The occurrence of the parasite in the skin ulcers, in discharges from the mouth and nose and in the fæces, renders direct or indirect infection possible. Dogs may act as reservoirs of the organism; but naturally infected dogs are not commonly found in India and artificial inoculations frequently fail to produce the disease in them. It is probable that an invertebrate host exists,

possibly a flea, bug, or sand fly (*Phlebotomus*), and that in it a sexual cycle occurs.

About the shores of the Mediterranean a very similar disease is found, except that it is confined almost entirely to children aged from two to five years. An organism (*Leishmania infantum*), which is identical morphologically with *L. donovani*, is found in the same situations in the

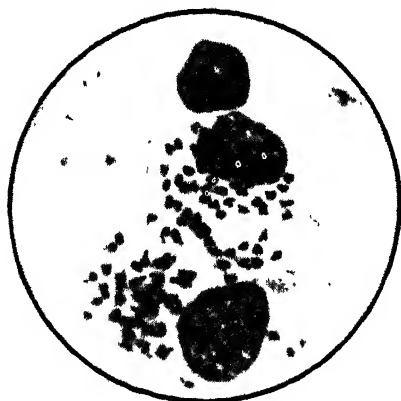


FIG. 92.—*LEISHMANIA TROPICA* IN SMEAR FROM ORIENTAL SORE ( $\times 950$ ).

body as is the latter parasite in kala azar. Many believe that *L. infantum* is really *L. donovani*, the virulence of which has been modified by passage. Where this disease occurs, many dogs are found to be naturally infected, and those unaffected are susceptible to inoculation. It is probable that in this case the dog acts as a reservoir and that the organism is transmitted by the sand fly, dog flea or other insect.

Diagnosis of the diseases may be made by spleen puncture which, if proper precautions are taken, is not dangerous in kala azar: liver puncture often reveals the presence of the parasites. Prolonged search of blood films may also show the Leishman-Donovan bodies, or these may be found in cultures of the blood. If enlarged lymphatic glands are present, one of them may be excised and the pulp examined.

The leucopenia, chiefly affecting the polymorphonuclear leucocytes, may assist diagnosis.

Another condition (Oriental sore, Bagdad button, or Delhi boil) is due to a similar organism. On an exposed part of the body a papule develops, this enlarges, becomes covered with scales and breaks down, leaving an ulcer. The ulcer enlarges and is unaffected by external treatment, healing only occurring after many months.

The organism producing the condition, *L. tropica*, is in almost all respects identical with *L. donovani*. It is found in the discharge or at the edges of the ulcer, usually within endothelial cells.

The disease is inoculable and is transmissible to dogs and monkeys, either by injection of the discharge or of cultures. Infection may be direct or indirect through the agency of flies or other insects. It is uncertain whether the parasite has a sexual cycle in the body of some invertebrate host such as *Phlebotomus*.

It is usually found that one attack confers a lasting immunity, and in animals experiments have shown a certain degree of immunity against *L. donovani* as a result of infection with *L. tropica* and vice versa. The treatment of Leishmaniasis has been greatly improved since the introduction of various antimony salts, which are administered intravenously. Of these the most satisfactory is "v. Hayden 471," which has greatly reduced the mortality and has also shortened the duration of treatment.

## CHAPTER XLVI

### PATHOGENIC FUNGI

CERTAIN pathological conditions either of the skin or, less commonly, of the subcutaneous tissues or of the viscera, have been found to be due to fungi. The most important of these resemble in many ways the moulds, but some are similar to the yeasts. In all these organisms we find that the morphology and methods of reproduction are of the simplest character while they are parasites, but when growing saprophytically, as on artificial media, the structure and mode of reproduction of many of them are complicated, and enable them to be grouped with the moulds commonly found in damp places under natural conditions.

Parasitic mycology is now a subject of considerable magnitude, and here it is impossible to give more than a brief outline of some of its most important points. A word as to the special methods involved is first necessary. In ringworm of the scalp the hairs to be examined should be taken from around the edge of the affected area. A convenient method of obtaining material from the nails is to scrape them with a microscope slide which has been broken, leaving a sharp edge. Epithelial scales from the skin may be detached with a forceps. For microscopic examination of the parasites in hairs and epithelial scales, the simplest method is to place the fragment in a drop of 40 per cent. potassium hydrate solution on a slide and cover with a cover slip. The slide is then gently warmed, and in a few moments hairs are ready for examination. A much longer time (two or three hours) is advisable in the case of epithelial scales. The alkali clears the tissue and the details of the fungus can be made out quite well. Staining methods are troublesome and not always satisfactory.

Many of the parasites can be cultured, and a suitable medium is agar containing 1 per cent. of peptone without

meat extract, but with 4 per cent. of either maltose or glucose. The reaction should incline to the acid rather than to the alkaline side of neutrality. Aerobic conditions are essential and a temperature somewhat below that of the body usually gives best results, although growth takes place either at air or at body temperature. One noteworthy feature of the cultures of these organisms is that very slight differences in the composition of the medium, even such as that found in two batches of media made according to the same formula, exercise great differences in the type of colony produced. For this reason it is difficult in many cases to recognize a fungus from the appearance or colour of the colony.

Ringworm is of two chief types, and these are due to the action of two different varieties of fungi. The ringworm which occurs almost exclusively in children, particularly on the scalp, is due to the *Microsporon*; that found in adults on the beard, hairless skin and nails and very rarely on the scalp, is caused by the *Trichophyton*.

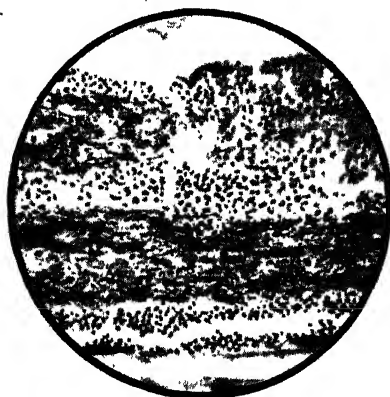


FIG. 93.—*MICROSPORON AUDOUINII* IN HAIR ( $\times 250$ ).

*Microsporon audouinii* (*Trichophyton microsporon*) is found in affected hairs and epithelial scales as filaments, which have a diameter of about 2 to 5  $\mu$ . These first appear in the superficial part of the epidermis, but soon they penetrate the hairs at the level at which they emerge from

their sheaths. The filaments, which grow along the hair downwards and for a short distance upwards, when carefully examined, are found to be branched. The filaments are not continuous, but their protoplasm is separated by cross divisions, which occur at some distance from one another. In many cases the hairs may show but slight signs of invasion,



since the most marked activity of the parasite is not in the interior but on the exterior of the infected hair which it covers. Towards the ends of branches the septa occur at closer and closer intervals, so that these branches end in a short chain of small, irregularly spherical spores measuring from 2 to  $3\mu$  in diameter. The whole of the exterior of the

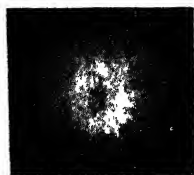


FIG. 94.—COLONY OF  
MICROSPORON AU-  
DOUINI ON GLUCOSE  
AGAR ( $\times 1$ ).

hair is covered with a layer composed of enormous numbers of these spores so tightly pressed together that they have assumed a polyhedral shape.

This parasite affects man exclusively, and occurs but rarely after the age of puberty. It has not commonly been found possible to infect either rabbits or guinea-pigs, and only a few successful inoculations have been recorded. There are, however, other varieties of microspora, which occur naturally in animals but are also pathogenic for man.

On glucose or maltose agar *M. audouini* grows well. The colonies, after about a fortnight, are large, of a white colour and covered with a delicate downy layer. In the centre there is usually a distinct elevation from which furrows pass towards the periphery, or occasionally an arrangement of concentric rings is seen. In cultures other methods of reproduction are observed—chlamydospores which are swollen, ovoid cells situated in the course of certain hyphæ, and conidia, small spores 2 to  $3\mu$  by 3 to  $4\mu$  arranged along the sides of hyphæ. Cultures of animal microspora may be distinguished from those of *M. audouini* by the occurrence of large spindle-shaped bodies measuring from 15 to  $20\mu$  by 40 to  $60\mu$ . These contain a number of cells and are situated at the free ends of hyphæ.

The large-spored ringworms, due to various varieties of *Trichophyton megalosporon*, affect man at all ages. They may cause disease in the beard, smooth parts of the body or in the scalp and nails. In these varieties there is more inflammation, and pus may be present. The parasites may be divided into two types—the endothrix, confined to the human race, in which the organism is found chiefly in the

interior of the hairs; and the endo-ectothrix types, which occur in man and animals, both in the interior and on the exterior of the hair. In a hair infected with a human trichophyton, branched mycelial filaments almost fill the hair; but in these the septa are so closely arranged that only chains of spores, which measure from 4 to  $6\mu$  in diameter, are seen. In the animal trichophyta the filaments are found both inside and around the hair. The spores in this case are more irregularly arranged.



FIG. 95.—TRICHOPHYTON ENDOTHRIX IN HAIR ( $\times 250$ ).

Different types of both human (*T. crateriforme*, *T. acuminatum*) and animal (*T. gypseum*, *T. felinum*, etc.) trichophyta may be distinguished by their cultural characteristics. The

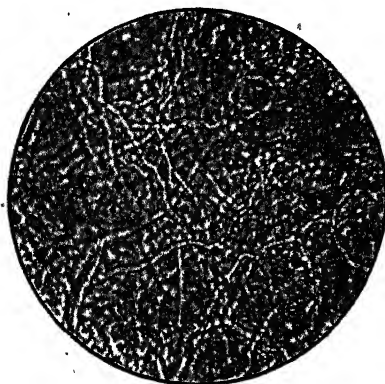


FIG. 96.—TRICHOPHYTON MEGALOSPORON IN SCALE OF SKIN ( $\times 250$ ).

colonies are usually white or grey, but in some a brown or violet colour may develop later. The central portion is elevated, with or without a small depression. Around the periphery delicate branches spread over the surface of the medium. The whole is covered with a fine downy layer. Some types show a marked arrangement of furrows running radially from the

centre towards the periphery. In cultures, the reproductive organs include conidia, which differ from those of microsporon,

since the spores are produced at the ends of short branches arranged along the course of a hypha, while in the microsporon

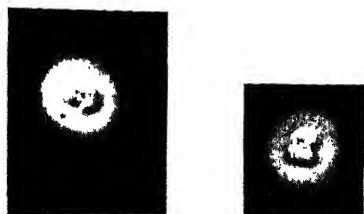


FIG. 97.—COLONIES OF TRICHOPHYTON ON GLUCOSE AGAR ( $\times 1$ ).

the conidia are attached directly to the hypha. Large spindle-shaped organs occur which resemble those of a microsporon, but the chlamydospores are round and not ovoid. In the trichophyta the terminal portion of certain hyphae is found to be arranged as a close spiral with from 8 to 10 turns. The object of these spiral filaments is unknown, but their occurrence is sufficient to distinguish a trichophyton from a microsporon.

Favus, which affects chiefly the scalp of children, is almost always due to the *Achorion schoenleinii*. A cup-shaped crust, the scutulum, which is of a yellow colour, forms about a hair. The scutulum is made up of a felted mass of fine septate, branching filaments, measuring from 2 to  $3\mu$  in diameter. In the central part the cross divisions approximate, leading to the production of rectangular spores. In the hair itself chiefly the spores are seen, and these show considerable variations in size.

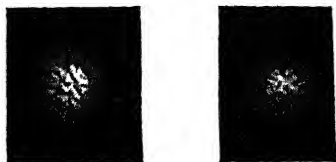


FIG 98.—COLONIES OF ACHORION SCHOLEINII ON GLUCOSE AGAR ( $\times 1$ ).

The colonies are of a grey or brown colour. They may be cup-shaped, but more usually show a warty or convoluted pattern. The edge is generally sharply defined and without the finely branching arrangement seen frequently in the microspora and trichophyta. The surface may appear to be covered with fine powder, but is not downy like the colonies of the ringworm organisms. In cultures, one of the most characteristic features is the occurrence of ovoid bodies of a yellow colour, situated usually

at the ends of the filaments and measuring about  $10\mu$  in diameter. Conidia are produced on the ends, or along the sides of some filaments. *Achorion schoenleinii* is only slightly pathogenic for animals. Of these the mouse is most easily infected, and in that animal a scutulum is produced. Other varieties, of which the most important is *Achorion quinckeanum*, occur naturally on other animals and are capable of infecting man.

Sporotrichosis is a rather rare condition in which granulomata are found, most commonly in the subcutaneous tissue and less often in the bones and viscera. The granulomata, which at first are hard and elastic, soften and break down, erupting on the surface through fistulæ from which serous pus is discharged. The lymphatic vessels become hard and thickened and along their course nodules may develop which also ulcerate. The lesions, which are very chronic, are commonly mistaken for those of syphilis or tuberculosis. In the discharge the causative organism, *Sporotrichum beurmanni* or *S. schenki* may be found either in the form of oval spores 3 to  $6\mu$  in length or, less commonly, as a mycelium.



FIG. 99.—COLONIES OF *SPOROTRICHUM BEURMANNI* ON GLUCOSE AGAR ( $\times 1$ )

In the majority of cases, however, no parasite is discovered microscopically in the discharge. For culture, the pus from an unopened abscess should be obtained and thickly spread on glucose or maltose agar slopes. The colonies, which at first are light in colour, darken to a chocolate brown as they get older. The surface is convoluted, and frequently the centre shows a depression. Around the periphery of the colony a white border is seen.

The serum of a patient suffering from sporotrichosis agglutinates the spores of the fungus in many cases up to  $\frac{1}{1000}$ . The presence of other fungi or of actinomyces in the body may cause a false agglutination, and for diagnosis a titre of not less than

$\frac{1}{200}$  is essential. The sporotricha are pathogenic for other animals—the mouse, rat and dog especially—and in them produce granulomata or multiple abscesses in the internal organs. Potassium iodide is an almost specific treatment for the condition.

Thrush is a disease chiefly of childhood, in which white patches occur on the mucous membrane of the mouth or

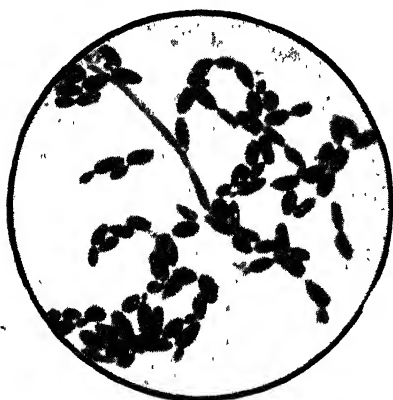


FIG. 100.—MONILIA FROM BROTH CULTURE ( $\times 700$ ).

pharynx. The patches are composed of the superficial epithelial cells and the causal organism, the *Oidium albicans* (*Monilia candida*). Two forms of the parasite are observed, round or ovoid cells, measuring from 5 to  $7\mu$  in diameter, which multiply by budding, and filaments composed of cells 4 to  $7\mu$  in diameter and about  $20\mu$  in length. The filamentous cells appear to be merely

modifications of the round or ovoid elements, since they also produce buds which may assume either the long or short form. On agar round, white, tough and adherent colonies are produced, in which practically only a short yeast-like form is seen; but in fluid cultures both the filamentous and yeast forms occur, as they do in the body. Probably there are a number of different fungi grouped together as one; some liquefy gelatin, others do not, and some possess marked fermentative powers resembling the yeasts, while others are inactive against carbohydrates. By scarification and inoculation of the mucous membrane of the mouth of some animals—the dog, cat and sheep—a condition resembling thrush may be produced. Subcutaneous inoculation of the rabbit leads to abscess formation. If intraperitoneal inoculation is performed a fatal sero-purulent peritonitis develops,

and a fatal generalized infection commonly follows intravenous injection.

A number of cases of infection in man by yeast-like bodies (*Blastomycetes*) have been recorded. Usually these have been confined to the skin, causing the development of small inflammatory nodules, which became abscesses, or of a lupus-like condition. More rarely abscesses of the bones, lungs or other viscera have been due to similar parasites. Some of these are probably true yeasts, multiplying by budding and actively fermenting carbohydrates. In others filamentous forms have been observed, and these are probably not to be grouped with the yeasts.



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